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ENZYMATIC DEGRADATION OF PRION PROTEIN BY KERATINASE PRODUCING PROTEOLYTIC MICROORGANISMS

Thesis submitted to Middlesex University London, UK in partial
fulfilment of the award of Doctor of Philosophy (PhD) Degree in
Environmental Science

By

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List of abbreviations

aa	amino acid
AHVLA	Animal Health and Veterinary Laboratories Agency
ANOVA	analysis of variance
ATCC	American Type Culture Collection
APS	ammonium persulfate
BF	Binding buffer fraction (fraction collected from passing binding buffer through purification column)
BH	brain homogenate
BS	crude biosurfactant extract
BSA	bovine serum albumin
BSE	bovine spongiform encephalopathy
BSM	basal mineral medium
CD	catalytic domain
CARD	Centre for Age Related Disease
°C	degree Celsius
CFU	colony forming units
CIBH	infectious brain homogenate control
CJD	Creutzfeldt-Jakob disease
CMC	critical micelle concentration
CNBH	normal brain homogenate control
CNS	central nervous system
CS	concentrated crude keratinase
CWD	chronic wasting disease
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EF	purified keratinase (fraction collected after eluting column with elution buffer)
EF+BS	purified keratinase + biosurfactant (enzymatic preparation)
ELISPOT	enzyme-linked immunospot assay
EMEM	Eagle's minimal essential medium
FMA	feather meal agar
FT-IR	Fourier transform infrared
g	gram
GPI	glycosyl-phosphatidyl inositol

GSS	Gerstmann-Strussler-Scheinker syndrome
h	hour
HCCA	α -cyano-4-hydroxycinnamic acid
HRP	horseradish peroxidase
IBH	infected brain homogenate
ID50	infectious dose
IF	intermediate filament
KA	keratin azure
KDa	kilo dalton
mA	milli ampere
mAb	monoclonal antibody
MALDI-TOF-MS	matrix-assisted laser desorption and ionisation time-of-flight mass spectrometry
MBM	Meat and Bone Meal
MGM	minimum growth medium
MWM	molecular weight marker
μl	microliter
μg	microgram
mM	millimolar
mg	milligram
ml	milliliter
min	minute
NBH	normal brain homogenate
NCIMB	National Collection of Industrial Food and Marine Bacteria
NCTC	National Collection of Type Cultures
ND	not determined
NIR	near infrared
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PD	propeptide domain
PFA	paraformaldehyde
pH	pondus hydrogenii
PK	proteinase K
PMCA	prion misfolding cyclic amplification
PMSF	phenyl methyl sulfonyl fluoride
ppm	parts per million

PrP^C	cellular prion protein
PrPres	proteinase K resistant prion protein
PrP^{Sc}	infectious prion protein
PSC	fraction collected after passing the concentrated crude supernatant through the column
PVDF	polyvinylidene fluoride
rDNA	ribosomal deoxyribonucleic acid
RNA	ribonucleic acid
rpm	rotation per minute
S	crude keratinase extract
SD1 and SD2	β-sandwiched domains
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEAC	Spongiform Encephalopathy Advisory Committee
SHEA	Society of Healthcare Epidemiology of America
sp	species
SRM	specified risk material
SSBP/1	sheep scrapie brain pool/1
SSCA	standard scrapie cell assay
TCA	trichloroacetic acid
TEMED	tetramethylethylenediamine
TME	transmissible mink encephalopathy
TNF-α	tumor necrosis factor
TSE	transmissible spongiform encephalopathies
TSI	triple sugar iron test
U	activity
UV	ultraviolet
V	voltage
vCJD	variant Creutzfeldt-Jakob disease
VP	Voges-Proskauer
WHO	World Health Organisation
WB	Western blot
WT	wild-type

Abstract

Prions are highly resistant to common proteases and conventional sterilisation processes. Consequently, prion infectivity is destroyed by methods such as incineration, alkaline and thermal hydrolysis. These harsh, destructive and potentially hazardous methods are unsuitable for processing specified risk materials (SRM) and animal by-products, and in the decontamination of medical and laboratory devices, and prion contaminated environments. Thus an environmentally friendly, enzymatic degradation and decontamination process is a highly desirable alternative. The structural similarity of prion and feather keratin suggests that feather degrading microorganisms have the potential to degrade prions. The objective of this research was to isolate and characterise microbial keratinase and to investigate its ability to degrade ME7 scrapie prion. Thirty two microbial strains were isolated on feather meal agar from primary effluent and farmyard wastes. One of the isolates, a Grams positive bacterium, demonstrated significant keratinolytic activity (11.00 ± 0.71 U/ml), and was investigated further. The isolate was identified by 16S rDNA and designated as *Bacillus licheniformis* N22, and was deposited in National Collection of Industrial Food and Marine Bacteria (NCIMB). The growth conditions for optimum keratinase synthesis in a minimal growth medium (MGM) were found to be pH 8.5, 50 °C, 1.1 % (w/v) feather meal substrate and at incubation time of 32 h. The molecular weight of purified keratinase was ≈ 28 KDa as measured by SDS-PAGE and confirmed by MALDI-TOF-MS. Optimum keratinase activity was obtained at pH 8.5 and 50 °C. This keratinase fully degraded recalcitrant melanised feather in 48 h, and also digested ME7 scrapie prion at 65 °C in 2 h to levels of PrP^{Sc} undetectable by western blot analysis. In a remarkable synergistic enzymatic preparation composed of keratinase and biosurfactant derived from *Pseudomonas aeruginosa* NCIMB 8626, ME7 scrapie prion was degraded to undetectable levels at 65 °C in 10 min. Interestingly biosurfactant alone showed no detectable activity on ME7 scrapie prion. Time-course degradation analysis showed progressive attenuation of PrP^{Sc} signal at 50 °C over time. Test of residual infectivity by standard cell culture assay showed that this enzymatic method completely destroyed standard sheep scrapie prion (SSBP/1) at 65 °C in 1 h. The mean survival time of mice challenged with enzyme-digested inocula significantly increased from 278 ± 9 days to 334 ± 42 days compared to those inoculated intraperitoneally with neat ME7 scrapie ($p = 0.008$ at 95 % confidence interval). Furthermore, 47 % of all the mice in enzyme-digested group lacked detectable levels of PrP^{Sc}. These results suggest a substantial reduction in the infectious titre or complete destruction of ME7 prion infectivity by the enzymatic preparation. Therefore, this mild enzymatic treatment method has potential applications for prion decontamination.

Chapter 1: Introduction

1.1 Transmissible spongiform encephalopathies (TSE)

Transmissible Spongiform Encephalopathies (TSEs) or prion diseases are a group of closely related, progressive, incurable and invariably fatal neurodegenerative disorders that affect the central nervous system (CNS) of mammals (Collinge, 2001). TSEs are broadly classified into the human and animal forms (Table 1.1). The human forms are categorised according to their aetiology into sporadic, acquired and inherited TSE groups, and the animal forms are described according to their natural host types (Table 1.1) (WHO, 1999; Collinge, 2001).

Table 1.1 TSEs classified according to the human and animal types, indicating the year it was first reported. The human types are categorised according to the aetiological groups and the animal forms according to their animal host types.

<i>Type of human TSEs</i>	<i>Aetiological groups</i>
1. Creutzfeldt-Jakob disease (CJD) (1921)	Sporadic disease <ul style="list-style-type: none"> No known antecedent event May occur by rare stochastic conversion of cellular prion protein
2. Kuru (1957) 3. Iatrogenic CJD (1974) 4. Variant CJD (vCJD) (1996)	Acquired disease <ul style="list-style-type: none"> Acquired by contamination with the infectious agent
5. Familial CJD (1924) 6. Gerstmann-Strussler-Scheinker syndrome (GSS) (1936) 7. Fatal familial insomnia (FFI) (1986) 8. Sporadic fatal familial insomnia (1999) 9. Atypical prion disease (APD)	Inherited disease <ul style="list-style-type: none"> Genetically inherited Specific gene mutations or insertion Different clinical and pathological features depending on the causative mutant gene
<i>Types of animal TSEs</i>	<i>Natural hosts</i>
1. Scrapie (1732)	Sheep and goat
2. Transmissible mink Encephalopathy (TME) (1947)	Mink
3. Chronic wasting disease (CWD) (1967)	Deer and elk.
4. Bovine spongiform Encephalopathy (BSE) (1986)	Cattle.
5. Feline spongiform Encephalopathy (FSE) (1990)	Domestic cats, puma, ocelot, tiger and cheetah.
6. Exotic ungulate encephalopathy (1986)	Identified in zoo animals e.g. kudu, gemsbok, nyala, eland, bison, ankole, Arabian oryx and scimitar.

TSEs are characterised by distinct clinical (e.g. rapid dementia, irritable demeanor, impaired movement and co-ordination, weight loss) and neuropathological (e.g. spongiform changes, amyloid plaque formation and accumulation of the prion agent) presentations (Prusiner, 1998; Collinge, 2001). Clinical signs in experimental rodents include truncal ataxia, forelimb flexion instead of extension when suspended by the tail, difficulty righting from a supine position, kyphosis (roundback), emaciation, lethargy, excitability, and generalised tremor and head bobbing (Prusiner *et al*, 2004; Pilon *et al*, 2009). There is currently no prophylaxis or therapeutic intervention for these diseases (Collinge *et al*, 2009). Some examples of physical presentation of TSE in infected mammals are presented in Fig. 1.1.



Scrapie in sheep



Kuru in human



BSE in cow

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Fig. 1.1: Physical presentations of TSEs in mammals, showing scrapie in sheep, Kuru in human and BSE in cow. The sheep scrapie is characterised by loss of wool due to scratching and the Kuru and BSE characterised by significant weight loss.

1.2 Nature of the infectious agent

The nature of the infectious prion agent responsible for TSEs remain a complex and controversial biological phenomenon described by a number of competing hypotheses such as the viral, protein and multi-component hypotheses.

1.2.1 Viral and Virinos hypotheses

The viral hypothesis postulates that the infectious agent is a virus or is virus-like in nature, and is genetically capable of coding its own proteins (Chesebro *et al*, 1985; Manuelidis *et*

al, 2009). The argument for a viral hypothesis was based on the fact that the agent is infectious and able to cause disease characteristic of viruses. Also small nucleic acid has been reportedly found in purified infectious scrapie sample (Kellings *et al*, 1994). However, the viral hypothesis is untenable because prion infectivity could not be destroyed by processes which ordinarily destroy viruses and nucleic acids such as nuclease digestion, autoclaving, UV and ionizing radiation (Alper *et al*, 1978; Prusiner, 1982; Alper, 1993; Jackson *et al*, 2005). Also, no TSE specific nucleic acid consistently associated with the prion agent has been identified (Prusiner, 1998). Besides, the infectious agent is not structurally similar to viruses.

The virinos hypothesis postulates that the infectious agent is a small informational molecule (mostly a piece of nucleic acid) which is too small to code its own protein (Dickinson and Outram, 1979), but which replicates by using the host made protein in which it is encapsulated (Kimberlin, 1982). Virinos are reported to withstand methods that destroy nucleic acid due to its small size and protective protein coat (Kimberlin, 1982). However, no nucleic acid has been found following nuclease digestion (Prusiner, 1998).

Despite the setbacks to the viral and virinos hypotheses, there are still proponents who argue that diversity in prion strains and the reported presence of small quantities of nucleic acid in infectious samples support these hypotheses (Chesebro *et al*, 1985; Narang, 2002; Manuelidis *et al*, 2009). However, heritable information defining distinct prion strains have been found not to reside on some informational molecule but rather exclusively in the protein folding pattern (Safar *et al*, 1998; King and Diaz-Avarios, 2004; Tanaka *et al*, 2004; Thackray *et al*, 2007). Therefore, the viral hypothesis remains unsubstantiated.

1.2.2 Prion hypothesis

Griffith (1967) first reported that the scrapie agent was probably a self-replicating protein. Subsequently, Stanley Prusiner and co-workers isolated the infectious agent by biochemical fractionation, and observed that it was inactivated by procedures that destroyed protein, suggesting that it was proteinaceous in nature (Prusiner, 1982; Bolton *et al*, 1982). Prusiner (1982) described the agent as **proteinaceous infectious** particle and named it '**prion**'. The prion (protein-only) hypothesis postulates that the infectious agent consists probably entirely of an abnormal protein conformer which is devoid of nucleic acid and yet able to replicate and propagate itself (Prusiner, 1982). Although the prion hypothesis was dismissed at the time as a biologically baseless aberration, a number of evidence supporting the hypothesis has accumulated over the years such as the discovery of the normal prion protein (Oesch *et al*, 1985) and prion protein encoding gene (Basler *et*

al, 1986), the effect of prion protein gene mutation on prion phenotyping (Hsiao *et al*, 1990), generation of infectivity by alteration of the prion protein (Sigurdson *et al*, 2009), the absence of other detectable components such as nucleic acids in a highly purified prion, which co-purify with infectivity, and which exhibit a proportionality of concentration to infectivity titre (Gabizon *et al*, 1988), the inability to propagate infectivity in prion protein knockout mice (Bueler *et al*, 1993; Legname *et al*, 2004), conversion of normal prion protein to prions in cell-free experiments seeded with infectious prion (Caughey *et al*, 1997) and in prion misfolding cyclic amplification (PMCA) (Saborio *et al*, 2001; Deleault *et al*, 2007), prion strain diversity resulting entirely from prion protein misfolding pattern (Prusiner, 1997; Caughey *et al*, 1998; Tanaka *et al*, 2004), generation of infectivity *in vitro* with natural and synthetic prion (King and Diaz-Avalos, 2004; Tanaka *et al*, 2004) and the presence of infectivity and transmissibility of amyloid forms of other protein misfolding diseases such as Alzheimer's, Parkinson diseases and Huntington disease, which provide evidence of protein-only transmission of biological information in the absence of nucleic acid (Cushman *et al*, 2010). Evidences for the prion hypothesis excludes possible pathologic and replicating role of small viruses, and clearly demonstrates that transmission of biological information and propagation of infectivity by protein in the absence of a nucleic acid template is possible. However, the prion hypothesis does not conclusively prove that prion is entirely the infectious agent, as clearly stated in its definition (Prusiner, 1982).

1.2.3 Multi-component hypothesis

The multi-component hypothesis postulates that the infectious agent may be composed of multiple host components including lipids, polyanionic molecules, cell adhesion molecules, RNA and glycosaminoglycan (Deleault *et al*, 2007; Geoghegan *et al*, 2007; Caughey *et al*, 2009; Wang *et al*, 2010). These cofactors or accessories may be required for the formation of infectious prion and the efficient replication and propagation of prion infectivity. For example, a decrease in prion generation and amplification occurred when purified prion protein was used as substrate instead of brain homogenate in *in vitro* experiment (Deleault *et al*, 2005). Similarly, *in vitro* conversion of normal prion protein to the infectious form using bacterially expressed hamster prion protein as substrate was less efficient than with brain homogenate (Atarashi *et al*, 2007). These examples suggest that additional components play an important role in the molecular events leading to the conversion, replication and propagation of prions.

While the prion hypothesis remains important and fundamentally relevant, the multi-component hypothesis is a complementing hypothesis which envisages broader

possibilities of the compositions and interactions of prions in a biological system. This is particularly important considering, for instance, that the presence of PrP or protease-resistant PrP alone may not result in prion conversion and propagation of prion infectivity. Therefore, the protease-resistant PrP may not entirely represent the infectious agent. However, despite the various hypotheses put forward, the nature of the infectious agent remains highly complex and poorly understood.

1.3 Prion protein

Prion protein is a membrane-bound glycoprotein first described in 1985 (Oesch *et al*, 1985), and which derives its name from the infectious agent ‘prion’ of TSEs (Prusiner, 1982). The term prion protein (**PrP**) generally refers to both the normal cellular form designated **PrP^C** and its pathological isoform designated **PrP^{Sc}**. The molecular weight of prion protein is 33-35 KDa.

1.3.1 Cellular prion protein (PrP^C)

PrP^C is a glycosyl-phosphatidyl inositol (GPI) anchored membrane protein encoded by the PrP gene (Basler *et al*, 1986; Stahl *et al*, 1987). In the intracellular milieu, PrP^C is endocytosed via the clathrin-coated pits and then recycled back onto the plasma membrane (Shyng *et al*, 1994). PrP^C is expressed in mammalian tissues and cells, and abundantly in the central nervous system (CNS) and lymphoid organs (Oesch *et al*, 1985; Antoine *et al*, 2000; Li *et al*, 2001). PrP^C has been detected in non-mammalian species such as birds (Gabriel *et al*, 1992), fishes (Rivera-Miller *et al*, 2003), chicken, turtle and frogs (Calzolari *et al*, 2005) and fungi such as *Podospira anserine* (Coustou *et al*, 1997).

1.3.1.1 Physiological functions of PrP^C

The evolutionarily conserved nature of the PrP gene (Oesch *et al*, 1991; Van Rheede *et al*, 2003; Wei *et al*, 2008; Crozet *et al*, 2008) and the expression of PrP^C in several mammalian and non-mammalian species, arguably suggest that it may play some important physiological role. However, the apparent lack of abnormal physical and physiological features in PrP^C knockout mice suggests that PrP^C is probably dispensable in physiological functioning (Bueler *et al*, 1992; Lipp *et al*, 1998).

The central role of PrP^C in prion pathogenesis has been demonstrated in cell-free conversion experiments in which PrP^C substrate was necessary for prion replication to occur (Caughey *et al*, 1997; Kirby *et al*, 2006), and in transgenic mice in which PrP^C over-expression resulted in shortened disease incubation time (Fischer *et al*, 1996; Tamguney *et al*, 2006). Also, PrP^C knockout mice were unable to propagate disease (Bueler *et al*, 1993;

Prcina and Kontsekova, 2011), which suggests that it plays important role in prion pathogenesis.

The possible roles of PrP^C in CNS and immune system functioning, for example in neuroprotection against apoptotic, oxidative and heat shock stress, trans-membrane signalling, memory formation and cognition and synaptic activity have been variously reported. PrP^C reportedly mitigated Bax-mediated cell death in human neurons and in yeasts (Bounher *et al*, 2006), and protected MCF-7 cancer cells exposed to apoptosis-inducing tumor necrosis factor (TNF- α) (Diarra-Mehrpour *et al*, 2004). It has been reported that cross-linking PrP^C with antibody resulted in the activation of extracellular-regulated kinase, NADPH oxidase and the production of reactive oxidase species, which suggests that PrP^C functions as a receptor for trans-membrane signalling (Mouillet-Richard *et al*, 2002). Also, PrP^C has been reported to play a role in memory formation and cognition (Criado *et al*, 2005).

Evidence of PrP^C role in immune system functioning has been shown by the abundant level of PrP^C expression in dendritic cells (Martinez *et al*, 2006). The absence of PrP^C in dendritic cells was found to result in a significant decrease in T cell response (Ballerini *et al*, 2006). Also, temporary inactivation of follicular dendritic cells delayed neuroinvasion of scrapie (Mabbott *et al*, 2000). Furthermore, PrP^C involvement in synaptic activity has been shown by its ability to bind Alzheimer's disease associated β -amyloid oligomer, and to act as a receptor militating against oligomer-induced synaptic dysfunction (Lauren *et al*, 2009). Also, PrP^C knockout mice were reported to show reduced sense of smell (judged by odour guidance test) compared to normal PrP^C expressing mice as a result of aberrations in the synaptic transmission between olfactory bulb granule and mitral cells (Le Pichon *et al*, 2009).

In general, the rather extensive list of possible functions of PrP^C arguably suggests that PrP^C is either a vastly important multi-functional physiological protein or one which actual function is clearly undefined.

1.3.1.2 Prion conversion and aggregation

The normal form of prion protein, PrP^C, is converted into an abnormal form, PrP^{Sc}, by the misfolding of α -helices of PrP^C into β -sheets (Pan *et al*, 1993; Prusiner, 1998). It has been demonstrated that the plasma membrane is the primary site of prion conversion (Goold *et al*, 2011). During this conversion, the single disulphide bonds that links and stabilises the helices on the C-terminal region of PrP^C remain intact (Huang *et al*, 1996; Herrmann and Caughey, 1998; Govaerts *et al*, 2004). The conversion and aggregation of PrP^C to PrP^{Sc} is

broadly described by two mechanistic models: the template-assisted model [heterodimer model] (Prusiner, 1991) and the nucleated polymerisation model [seeded fibrillisation] (Jarret and Lansbury, 1993; Birkman and Riesner, 2008).

The template-assisted model suggests that PrP^{Sc} serves as a template by which $\text{PrP}^{\text{C}} / \text{PrP}^{\text{Sc}}$ complex results in the conversion of PrP^{C} to PrP^{Sc} (Fig 1.2A). The $\text{PrP}^{\text{C}} / \text{PrP}^{\text{Sc}}$ complex suggestively lowers high energy barrier that would ordinarily impede spontaneous PrP^{C} to PrP^{Sc} conversion (Aguzzi *et al*, 2008). The template-assisted model is exemplified by the cell-free conversion experiment in which radioactive labelled PrP^{C} molecules seeded with PrP^{Sc} molecules result in radioactive labelled PrP^{Sc} molecules (i.e. radioactive labelled $\text{PrP}^{\text{C}} + \text{PrP}^{\text{Sc}} = \text{PrP}^{\text{Sc}} + \text{radioactive labelled PrP}^{\text{Sc}}$) (Caughey *et al*, 1997).

The nucleated polymerisation model suggests that PrP^{C} and PrP^{Sc} are in a reversible thermodynamic equilibrium, co-existing in normal state, with the monomeric PrP^{Sc} having a tendency to aggregate into infectious PrP^{Sc} seed. The infectious seed then recruits more monomeric PrP^{Sc} at a faster rate and aggregates into amyloid, and the amyloid then fragments into more infectious seeds and so on (Fig 1.2B). This model highlights that PrP^{Sc} aggregation is required in prion pathogenesis. The model is exemplified by the *in vitro* conversion of PrP^{C} to PrP^{Sc} in protein misfolding cyclic amplification (PMCA) assay, where the PrP^{Sc} aggregate is fragmented by sonication yielding additional PrP^{Sc} as template for PrP^{C} conversion (Caughey *et al*, 1997; Saborio *et al*, 2001). *De novo* generated PrP^{Sc} has shown replicating and pathologic properties as evidenced by *in vivo* propagation and neurological features (Castilla *et al*, 2005; Barria *et al*, 2009).

Despite the seeming competence of PrP^{Sc} to conscript and convert PrP^{C} , it has been suggested that other “partners in crime” are necessary for the conversion to occur (Caughey and Baron, 2006). A number of reports suggest that some hypothetical cofactors macromolecules such as glycosaminoglycans (Caughey *et al*, 1994; Hijazi *et al*, 2005; Silva *et al*, 2010), nucleic acids (Cordeiro *et al*, 2001; Deleault *et al*, 2003; Cordeiro *et al*, 2005; Silva *et al*, 2008), and extracellular matrix molecules (Leucht *et al*, 2003; Qiao *et al*, 2009) are involved in the efficient structural conversion process from PrP^{C} to PrP^{Sc} , and indeed in prion pathogenesis. These cofactor molecules supposedly lower the Gibbs free energy barrier making unfolding, oligomerisation and spontaneous conversion of PrP^{C} to PrP^{Sc} possible (Prusiner, 1998; Baskakov *et al*, 2004; Cordeiro and Silva, 2005). An example of the role of cofactors has been shown in cells, in which conversion and propagation of PrP^{Sc} was hindered by limiting the availability of cellular heparin sulphate (a glycosaminoglycans occurring in the cell membrane of most cells) (Ben Zakan *et al*,

2003; Hijazi *et al*, 2005). Despite the current knowledge of PrP^{C} interactions and PrP^{Sc} formation, the underlying mechanisms for prion conversion remain unresolved.

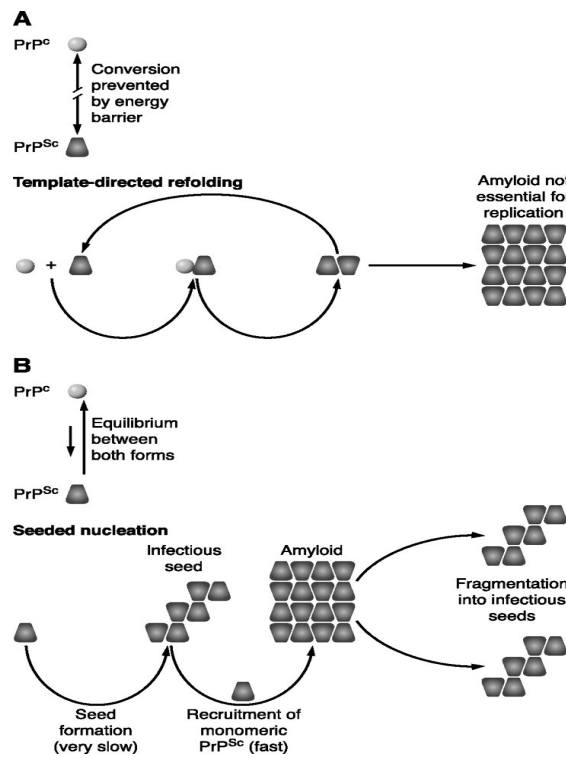


Fig. 1.2: Models for the conformational conversion of PrP^{C} into PrP^{Sc} . A: the template assistance model postulates an interaction between exogenously introduced PrP^{Sc} and endogenous PrP^{C} , which is induced to transform itself into further PrP^{Sc} . B: nucleation (Adapted from Aguzzi and Calella, 2009).

1.3.2 Infectious prion protein (PrP^{Sc})

Infectious prion protein (PrP^{Sc}) derives from normal cellular prion protein (PrP^{C}) after undergoing a post-translational conformational change, resulting in predominantly high β -sheet content in its secondary structure (Pan *et al*, 1993; Prusiner, 1998). PrP^{Sc} is considered to be the main component of the infectious prion agent (Prusiner, 1982), and its misfolded pattern determinant of prion strain diversity (Prusiner, 1997; Caughey *et al*, 1998; Tanaka *et al*, 2004). PrP^{Sc} is partially digested by proteinase K (PK), resulting in a PK resistant core (PrPres) consisting of amino acid residues 90 to 231, and molecular weight (MW) of approximately, 27-30 KDa (Prusiner *et al*, 1983; McKinley *et al*, 1983).

1.3.2.1 Prion infectivity

Prion infectivity is the characteristic of the prion agent that permits invasion, survival and replication, resulting in disease in a susceptible host. PrP^{Sc} and particularly its protease resistant core (PrPres) are generally considered as a surrogate marker for prion infectivity and disease. In principle, if PrP^{Sc} and PrPres constitute the main component of the infectious agent, their decreased concentration would almost certainly result in a

corresponding reduction in infectivity (Triantis *et al*, 2007). While a number of reports have shown strong correlation between PrP^{Sc} and infectivity (McKinley *et al*, 1983; Prusiner, 1991; Wadsworth, 2007), it has also been reported that PrP^{Sc} or PrPres does not always correlate with infectivity (Berardi *et al*, 2006; Barron *et al*, 2007; Lasmezas *et al*, 1997; Tixador *et al*, 2010; Silveira *et al*, 2005; Edgeworth *et al*, 2009). For example, Somerville *et al* (2002) reported a >99.9 % loss of infectivity after heat treatment of scrapie at 100 °C whereas PrP^{Sc} abundance persisted. On the contrary, infectivity has been reported in the absence of immunochemically detectable levels of PrP^{Sc} (Berardi *et al*, 2006; Barron *et al*, 2007; Lasmezas *et al*, 1997). Also, protease-sensitive and soluble forms of PrP^{Sc} have been reported to be potentially infectious, so that the absence of detectable PrPres does not necessarily indicate absence of infectivity (Berardi *et al*, 2006).

The variable reports of the relationship between prion infectivity and PrP^{Sc} cast doubts as to their actual relationship, and whether PrP^{Sc} entirely constitutes or indicates the presence of the infectious agent. Scherbel (2007) argues that the inability to detect PrP^{Sc} in prion infected brain homogenate may be as a result of low levels of PrP^{Sc}, presence of sub-fractions of infectious prion protein undetectable by immunochemical method and presence of other infectious molecules or structure other than PrP^{Sc}, and therefore alternative markers of prion infectivity and more sensitive PrP^{Sc} detection methods are required. However, PrP^{Sc} and PrPres remain important and established biomarkers of prion infectivity and prion diseases. For example, a reduction or loss of PrP^{Sc} immunoreactivity detected by Western blotting most likely suggests a reduction or loss of infectivity, and hence Western blotting analysis could be a useful biochemical method for detection of PrP^{Sc} and evaluation of residual infectivity (Caughey *et al*, 1997; Triantis *et al*, 2007; Wadsworth, 2007). Therefore, procedures that destroy PrP^{Sc} and methods that measure and quantify PrP^{Sc} are central to prion degradation and residual infectivity evaluation.

1.3.3 Structure and composition of prion protein

Full length PrP^C consists of two structurally well defined domains: a long amino-proximal (N-terminal) and carboxyl-proximal (C-terminal) domains (Fig. 1.3) (Aguzzi and Calella, 2009). The N-terminal domain consists of the signal peptide (1-22 aa residues) which is usually cleaved during PrP^C metabolism, and a flexible random coil sequence (23-124 aa residues) which contains several highly conserved metal-binding octapeptide repeats bordered by two positively charged clusters, CC₁ (aa 23-27) and CC₂ (aa 95-110) (Aguzzi and Calella, 2009). The metal binding property of octapeptide repeats is implicated in transformation and alteration of PrP^C biochemical properties leading to PrP^C stability and resistance to proteolysis, and may play a role in prion misfolding and prion pathogenesis

(Lehmann, 2002; Jackson *et al*, 2001; Choi *et al*, 2010). The N-terminal domain also contains a large bouquet of N-glycans attached to the PrP^C molecules (Rudd *et al*, 1999), and sulphated glycans binding to the structures of this domain was reported to modulate PrP^C conformational conversion and oligomerisation (Taubner *et al*, 2010), hence is central to PrP^C function and prion pathogenesis (Laffont-Proust *et al*, 2006). The N- and C-terminal domains are separated by a highly conserved hydrophobic segment (111-134 aa residues) (Aguzzi and Calella, 2009).

The C-terminal domain is globular in nature and consists of a GPI anchor which results in cleavage of membrane anchor region (aa residues 231-254) during its attachment to PrP^C, and a disulphide bond linking aa 178 and aa 213 which stabilises the domain. It also consists of two N-linked glycosylation sites in asparagine (asn) residues 181 and 197 (in human) and 180 and 196 (in mice) to which are attached different sugar types (Rudd *et al*, 2001). The glycosyl groups confer PrP^C with three different glycosylation forms namely unglycosylated, monoglycosylated and diglycosylated bands (Endo *et al*, 1989), which appear as lower, middle and upper bands respectively on PrP electrophoretic profiles (Parchi *et al*, 1996). It has been reported that the second helix of the C-terminal and the unstructured N-terminal regions (Fig 1.4) play a critical role in pathogenic conformational conversion of PrP^C (Ji and Zhang, 2009).

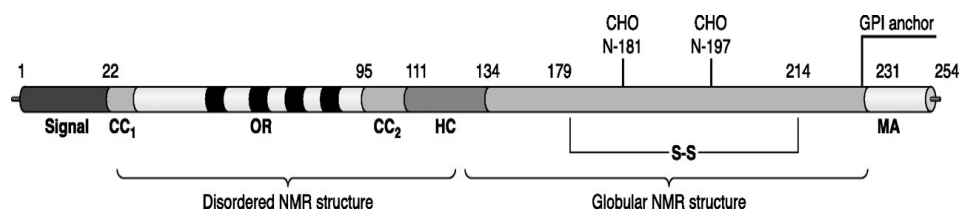


Fig. 1.3: Outline of the primary structure of the cellular prion protein including post-translational modifications. Where, signal: signal peptide residue; CC₁ and CC₂: positively charged clusters; OR: octapeptide repeats; HC: conserved hydrophobic segment; S-S: single disulphide bond linkage; CHO: N-linked glycosylation sites; GPI: glycosyl-phosphatidyl inositol anchor; MA: Membrane anchor region (Adapted from Aguzzi and Calella, 2009).

The cellular prion protein (PrP^C) and the pathogenic form (PrP^{Sc}) are indistinguishable in their primary amino acid composition, but characteristically differ in their secondary conformations resulting from their α -helical (spiral form) and β -sheet (plane form) compositions. Nuclear magnetic resonance (NMR) studies suggest that the secondary structure of PrP^C (Fig. 1.4) consists of three α -helices (aa residues 144 -154, 172- 193 and 200- 227) and two very short β -sheets (aa residues 129-131 and 161-163), and a relatively unstructured N-terminus (Riek *et al*, 1997; James *et al*, 1997).

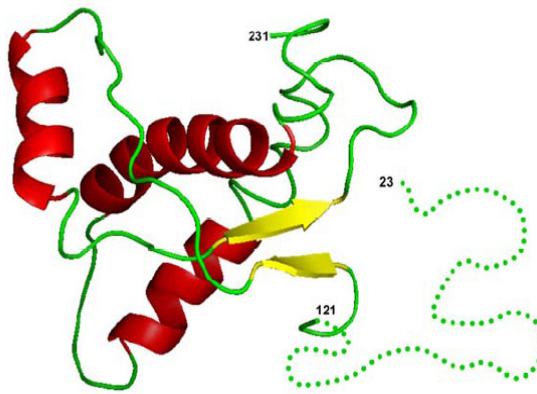


Fig. 1.4: Secondary structure of mouse prion protein showing three α -helices (represented in red) and two very short β -sheets (represented in yellow). Dotted line is unstructured N-terminus region (Adopted from Riek *et al*, 1997). PrP^C and PrP^{Sc} are differentiated by the amount of α -helices and β -sheets present.

Fourier-transformed infrared and circular dichroism spectrometry studies suggest that PrP^C is composed of approximately 42 % α -helical and 3 % β -sheet conformations, whereas PrP^{Sc} is composed of approximately 30 % α -helical and 43 % β -sheet conformation (Pan *et al*, 1993; Prusiner, 1998). The ratios of the α -helical and β -sheet content confer unique biological, biochemical and biophysical properties to both isoforms (Table 1.2). Clearly, high β -sheet conformation is an important characteristic of PrP^{Sc} and the primary building block of prion. The high β -sheet content in PrP^{Sc} results from the conformational transition of the hydrophobic region (amino acid residue ~90-140) of the PrP^C helices resulting in its characteristic hydrophobic nature and resistance to proteolysis and common physical and chemical processes (McKinley *et al*, 1991; Taylor *et al*, 1999). It has been suggested that proteins with high β -sheet content are kinetically stable and extremely resistant to proteolysis by PK probably due to their rigid structure (Manning and Colon, 2004). The implications of a structurally stable and protease resistant infectious prion include perseverance in contaminated environments and materials, and the attendant risks of potential transmission within and between species (Georgsson *et al*, 2006; de Pedro-Cuesta *et al*, 2009).

PrP^{Sc} is structurally similar to feather keratins by the prevalent nature and composition of high β -sheet abundance, which has become the basis for the hypothesis that feather degrading proteases such as keratinases are able to degrade prions (Shih, 2002; Langeveld *et al*, 2003) (see section 1.8).

Table 1.2: Biochemical and biophysical characteristics of PrP^C and PrP^{Sc}

Properties	PrP ^C	PrP ^{Sc}
Protein ^{1,2}	Normal cellular protein	Abnormal disease-associated protein
Infectivity ^{1,2}	Not infectious	Infectious, pathogenic
Folding ^{3,4}	Predominantly α -helices	Predominantly β -sheets
Solubility ⁵	Soluble (Hydrophilic)	Insoluble (hydrophobic)
PK digestion ⁶	Sensitive	Partially resistant
Aggregation ^{6,7}	Not aggregated	Tendency to aggregate

Oesch *et al* (1985)¹, Prusiner (1982)², Pan *et al* (1999)³, Prusiner (1998)⁴, Mckinley *et al* (1991)⁵ Prusiner *et al* (1983)⁶, Riesner (2003)⁷

1.4 Keratins: structure and composition

Keratins are highly stable, insoluble fibrous proteins composed of tightly packed α -helix (α -keratin, e.g. hairs) or β -sheet (β -keratin, e.g. feathers) intertwined polypeptide chains which form dense network of intermediate filaments (IF) (Fig. 1.5). The intertwined polypeptide chains consist of helical rod domain and a structurally short non-helical head (amino) and tail (carboxyl) domains (Cohlberg, 1993). Keratins belong to type I (acidic keratins) and type II (basic keratins) intermediate filament proteins and have molecular weights of approximately 45 KDa and 55 KDa respectively. There are approximately 30 different keratins which are generally grouped into epithelia keratins (in epithelia cells) and trichocytic keratins which make up hair, nails, horns and reptilian scale. Keratins are also classified as hard (those containing up to 5 % sulphur) and soft (those containing up to 1 % sulphur) keratins (Karthikeyan *et al*, 2007).

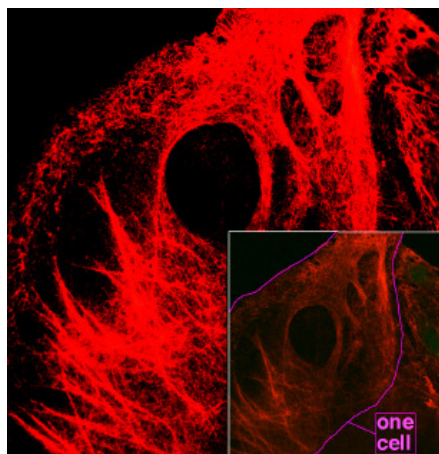


Fig. 1.5: Microscopic details of dense network of keratin filaments in a cell (308 × 310 pixels). Image by Jamie Bush and John Schmidt (2006) (Source: <http://en.wikipedia.org/wiki/Keratin>, 8/9/09).

Keratins consist of large proportion of the smallest amino acids such as glycine (with a side group of a single hydrogen atom) and alanine (with a small and non-charged methyl group). The stability of keratin depends on the composition and molecular conformation of its constituent amino acids (Bradbury, 1973), and on their intermolecular disulphide bond cross-linking (Wang *et al*, 2000). Hydrogen bonding between the amino and carboxyl groups of the peptide bonds on adjacent protein chains facilitates close alignment and strong binding in β -keratins (Bradbury, 1973). The tertiary structure of β -keratin is richly organised and stabilized by a high degree of cross-linking of disulfide bonds, and by hydrogen bonds and hydrophobic interactions (Murayama-Arai *et al*, 1983; Fuchs, 1995; Parry and North, 1998). These bonds together with its aggregated amyloid-like fibrillar structure confer keratin with its mechanical strength as well as chemical and enzymatic resistance (Alexander and Hudson, 1954; Fuchs, 1995). The highly stable and resilient nature of keratins is probably the reason why it is the main component of the epidermis and its appendages such as hair, nail, hoof, scale and feather. Fourier transform infrared (FT-IR) and near infrared (NIR) suggest that β -sheet structure are stabilised by formic acid (Aluigi *et al*, 2007). However, it has not been reported whether the β -sheet structure of prion is similarly stabilised by formic acids or indeed any other such entities.

Significant amount of keratins in the form of feathers, horns, hairs and hooves are generated as waste products from poultry, slaughterhouse, and leather processing industries (Onifade *et al*, 1988; Karthikeyan *et al*, 2007) which can be processed into protein rich livestock feed, fertilizers (Odetallah *et al*, 2003; Karthikeyan *et al*, 2007). Feather is composed of approximately 90 % pure keratin and consists predominantly of β -keratin (Fraser *et al*, 1972). Feather keratin (β -keratin) is highly stable due to its rich β -sheet constitution (Kelly *et al*, 2006; Kreplak *et al*, 2004), and hence most resistant to proteolysis (Fuchs, 1995). For this reason, it is used as a model substrate for the study of keratin degradation and as a substrate for routine study of hard-to-degrade proteins such as prions (Suzuki *et al*, 2006). Proteolytic microorganisms possessing feather degrading ability are considered as potential candidates for degradation of prions. The isolation and evaluation of prion degrading microorganisms and their proteases by procedures and assays that exploit their keratin degrading potentials is both safe and desirable, and was therefore explored in this study.

1.4.1 Degradation of keratin

Despite the stable structural composition of keratin, they are degraded by reducing agents such as thioglycolic acid, potassium cyanide, and sodium sulphide and sodium sulphite,

which rapidly degrade keratin under alkaline conditions by breaking the disulphide bonds that hold keratins together (Goddard and Michaelis, 1934). Also, oxidising agents such as bromine, permanganate and hydrogen peroxide slowly cleave keratins but not by breaking the disulphide bonds (Goddard and Michaelis, 1934). This therefore suggests that chemical degradation of keratins may occur by more than one mechanism.

The major setbacks of chemical degradation of keratins, especially in large scale technological applications is that the keratinaceous material may be completely destroyed and polluting chemical by-products could impact adversely on the environment. For example, the chemical processing of hide in the leather industry using sodium sulphide, lime and other solvents result in offensive odour and pollution of tannery effluents (Kamini *et al*, 2010). These limitations have warranted considerable interest in microbial enzymes as viable alternative technology which could allow product recovery but with significantly reduced pollution load. For example, keratinases which hydrolyse keratin can be used for skin dehairing in the leather processing industry without the chemical pollution associated with sodium sulphide treatment (Macedo *et al*, 2008; Tiwary and Gupta, 2010).

Degradation of keratin by certain specialised microorganisms have long been recognised as evidenced by the decomposition of feather keratin by some naturally occurring biological process in the environment (Noval and Nickerson, 1959); during which it is utilised as source of carbon and energy for growth and survival (Riffel and Brandelli, 2006; Joshi *et al*, 2007). Goddard and Michaelis (1934) reported that powerful protease and an enabling highly alkaline condition were a prerequisite for cleavage of disulphide bonds of keratins.

A prototypical protease highly efficient in keratin degradation is proteinase keratin commonly abbreviated as proteinase K (PK). PK is a broad spectrum serine protease commonly used to digest protein in molecular biology and for characterisation of prion protein by virtue of its ability to completely degrade PrP^C and partially degrade PrP^{Sc} (Prusiner *et al*, 1983; McKinley *et al*, 1983; Cronier *et al*, 2008; Safar *et al*, 2005; Tzanban *et al*, 2002; Pastrana *et al*, 2006). The general reaction mechanism for substrate degradation described for PK involves the attachment of the substrate to the substrate-recognition site in the active site, formation of hydrogen bonds in the oxyanion hole and hydrolytic cleavage by the catalytic triad (Betzel *et al*, 1988). The ability of PK to degrade both native keratin and prions (Shih, 2002; Langeveld *et al*, 2003; Tsirolnikov *et al*, 2004) may relate to their similar structural composition, suggesting that they may be degraded by similar mechanisms. It is for this reason that β -sheet rich keratinaceous materials

are suitable non-infectious substrate for routine evaluation of prion degrading proteases such as keratinases.

1.4.1.1 *Keratinases*

Keratinases are a group of proteolytic enzymes with the ability to hydrolyze insoluble protein keratin more efficiently than other proteases (Onifade, 1998). Keratinases have also been defined as proteolytic enzymes that catalyse the cleavage and hydrolysis of keratins (The American Heritage Medical Dictionary, 2007). In all, keratinases are proteases involved in the degradation of keratins by hydrolysis of its constituent polypeptide substrates into smaller molecular units which are readily absorbed by the microbial cells resulting in organic matter turnover (Cohen, 1990).

Keratinases are produced by several microbial species including fungi (Kushwaha and Gupta, 2008; Bahuguna, and Kushwaha, 1989; Gradisar *et al*, 2005), actinomycetes (Gusterova *et al*, 2005; Mitsuiki *et al*, 2010; Montero-Barrientos *et al*, 2005) especially the *Streptomyces* genus (Korkmaz *et al*, 2003; Bockle *et al*, 1995; Chao *et al*, 2007; Esawy, 2007; Bressollier *et al*, 1999; Tapia and Simoes, 2008) and *Bacillus* species (William *et al*, 1990; Ramnani *et al*, 2005; Manczinger *et al*, 2003; Olajuyigbe and Ajele, 2005) and archaea (Kublanov *et al*, 2009). Recently, keratinases from *Pseudomonas aeruginosa* KS-1 (Sharma and Gupta, 2010) and *Pseudomonas* sp. MS21 (Tork *et al*, 2010) have also been reported. This is particularly interesting as *Pseudomonas aeruginosa* are prolific producers of biosurfactants known to enhance degradation of recalcitrant organic matter such as hydrocarbons (Noordman and Jassen, 2002; Soberon-chavez *et al*, 2005; Okoroma, 2006) [see section 1.7].

Bacillus licheniformis are the most prolific keratinase producing bacteria and their proteases are known to be highly stable and significantly retain their activity in environments containing potentially inhibitory chemicals agents such as surfactants, magnesium and calcium ions, and sodium tripolyphosphate (Veith *et al*, 2004). Keratinase producing microbial species are isolated from various environmental sources including: soil (Korkmaz *et al*, 2003; Macedo *et al*, 2005), feather waste (Cai *et al*, 2008; Wang and Shih, 1999; Leterneou *et al*, 1998; Manczinger *et al*, 2003), poultry plant waste (Tapia *et al*, 2008), sea shore Esawy (2007) and cement of bathroom tile (Mitsuiki *et al*, 2010).

Keratinases are characterised by their biochemical and biophysical properties (Goldstein, 2007; Brandelli *et al*, 2010). Most keratinases thrive in alkaline and thermophilic environments (Williams *et al*, 1990; Bressollier *et al*, 1999; Korkmaz *et al*, 2003). The

molecular weight of reported keratinases is around 18 KDa and 280 KDa (Brandelli *et al*, 2010). Keratinases are grouped according to their ability to hydrolyse specific substrates and their activity in the presence of certain chemical agents. Serine keratinase group is the most commonly found and is characteristically inhibited by phenylmethylsulfonyl fluoride (PMSF) and benzamidine (Giongo *et al*, 2007). The metalloproteases group are inhibited by ethylene diamine tetra acetic acid (EDTA) (Thys and Brandelli, 2006; Riffel *et al*, 2007), and the serine metalloprotease keratinase are inhibited by both PMSF and EDTA (Tatineni *et al*, 2008).

The chemical structure of *Fervidobacterium pennivorans* keratinase, Fervidolysin, mapped by crystallographic analysis (Fig 1.6) suggests that it is composed of four domains; a catalytic domain (CD), propeptide domain (PD), and two β -sandwiched domains (SD1 and SD2) held together by hydrogen bonds and hydrophobic interactions (Kim *et al*, 2004). It is further suggested that keratinases of molecular weight less than 35 KDa may contain only the catalytic domain (Kim *et al*, 2004).

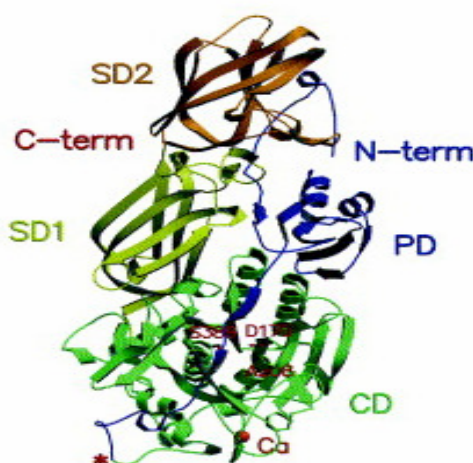


Fig.1.6: A 1.7Å resolution crystal structure of keratinase showing four domains: Catalytic domain (CD), propeptide domain (PD), and two β -sandwiched domains (SD1 and SD2), labelled according to corresponding colours. The active site catalytic triad consists of Ser389, His208 and Asp170. The PD-processing site at the boundary of PD and CD is indicated by red asterisks and calcium-binding sites indicated by balls labelled Ca. (Kim *et al*, 2004).

Considerable attention has been drawn to keratinases in recent years for their ability to degrade recalcitrant and environment polluting keratin wastes (feathers, hairs, and hooves) into useful end products. For example, keratinases convert feather wastes into nutritionally rich livestock feed (Chandrasekaran and Dhar, 1986; Shih and William, 1990; Onifade *et al*, 1998; Odetallah, *et al*, 2003) and slow-releasing organic fertilizers (Choi and Nelson,

1996). They are also becoming increasingly important for dehairing of hide in the leather processing industry (Mukhopadhyay and Chandra, 1993; Macedo *et al*, 2005; Tiwary and Gupta, 2010) and for degrading prions (Shih, 2002; Langeveld *et al*, 2003; Tsiroulis *et al*, 2004; McLeod *et al*, 2004; Miwa *et al*, 2006). Keratinase of *Bacillus licheniformis* PWD-1 is one of the famously reported prion degrading enzyme, and was found to degrade prions more efficiently than PK when tested at the same levels of enzymatic units (Chen *et al*, 2005). However, the quest for prion degrading proteases has intensified in recent years (Muller-Hellwig *et al*, 2006; Yoshioka *et al*, 2007), and recently, genetically modified prion degrading proteases have been reported (Dickinson *et al*, 2009; Liang *et al*, 2010). The uses and potential applications of keratinases summarised according to their industrial applications (Brandelli *et al*, 2010) is presented in Table 1.3.

Table 1.3: Potential applications of microbial keratinases grouped according to applications in different fields.

Field	Applications
Agro industry	<ul style="list-style-type: none"> - production of food hydrolysates - feed supplements - production of entomopathogens - production of nitrogen fertilizers
Pharmaceutical/ biomedical	<ul style="list-style-type: none"> - <i>prion hydrolysis</i> - enhanced drug delivery - dermatological treatment - cosmetics
Industry	<ul style="list-style-type: none"> - leather processing (Tannery) - fibre modification (Textile) - detergents - modification of protein functionality (foods) - biopolymers (Films, coating, glues) - wastewater treatment/waste management
Biomass/bioenergy	<ul style="list-style-type: none"> - bioconversion of keratinous wastes for Subsequent production of methane and biohydrogen.

Source: Brandelli *et al* (2010)

1.4.1.2 Mechanisms of keratin degradation by keratinase

The tight structural arrangement of keratin hinders proteolytic access to its cleavage sites such that it is degraded only by a few proteases, specifically keratinases. The structural configuration of keratinases enables the interaction with β -keratin and assist in disassembling of the layers of β -structures so that individual strands can be subsequently hydrolysed (Kim *et al*, 2004). The specific mechanism of keratin degradation by keratinase is still not well elucidated but appears to occur in two main steps: deamination and cleavage of disulphide bond. Deamination creates an alkaline environment needed for substrate swelling, sulphytolysis and proteolytic attack (Kunert, 1989; Kunert, 1992;

Kunert, 2000), where sulphytolysis is likely an essential step in the digestion of keratin which precedes proteolysis (Monod, 2008). Deamination is therefore considered as the first step in keratin degradation and results in the pH increases observed during feather degradation by microorganisms of significant keratinolytic activity (Kunert, 2000; Kaul and Sumbali, 1997). The second step in the degradation process involves cleavage of the disulphide bonds which changes the conformation of keratin therefore exposing more sites to proteolytic attack (Gradisar *et al*, 2005; Cao *et al*, 2008). The release of soluble sulfhydryl compounds is regarded as an indication of disulphide cleavage during microbial decomposition of keratin (Goddard and Michaelis, 1934). However, Williams *et al* (1990) reported that keratinase degradation of keratins occurred without an accompanying release of soluble sulfhydryl, which suggests that the degradation possibly follow different pathways, depending on the type and origin of the keratinase and the prevailing degradation conditions. Therefore, keratinases may show variable activity towards different keratin substrates as well as different prion strains.

Degradation of keratin by keratinases may be enabled in the presence of reducing agents. Ramnani and Gupta (2007) reported that suitable reducing agents e.g. live cells or chemical reductants are required in the degradation of feather keratins by keratinases. Also, it has been reported that other enzymes such as reductases may be constitutively secreted with keratinases in fermentation culture (Yamamura *et al*, 2002; Esawy, 2007; Manczinger *et al*, 2003; Rodziejewicz and Laba, 2008; Brandelli, 2008), or the presence of microbial consortium (Ichida *et al*, 2001) are required in the degradation of keratin in which case each enzyme or member organism contributes toward the degradation process perhaps via different pathways. However, it could be argued that the degradation of keratins by purified keratinases indicates that a reducing agent, multiple enzymes or indeed a microbial consortium may not necessarily be required for keratin degradation to occur (Lin *et al*, 1992; Bressolier *et al*, 1999; Anbu *et al*, 2005), but could play a role in stimulating enzyme activity resulting in enhanced degradation rate.

Considering that keratinases selectively degrade β -keratin by processes that break the stabilising bonds of the β -sheet structure (Burt and Ichida, 2001; Fuchs, 1995), it is reasonable to hypothesise that the β -sheet structure of prions can be degraded by similar degradation mechanisms.

1.5 Challenges of prion contamination

1.5.1 Risks of medical therapy

The BSE epidemic in the UK (1989-1999) raised significant public awareness of TSEs. As at 1999, the number of confirmed BSE cases was 176,299 (AHVLA, 2011), and approximately 2 million cattle were estimated to have been potentially infected with BSE (Smith and Bradley, 2003). In addition, approximately 1.5 million potentially infected cattle were estimated to have passed into the human food chain and potentially consumed by millions of UK residents (Donnelly *et al*, 2002). Epidemiological and laboratory evidence of a causal link between BSE and vCJD (Brown *et al*, 1987; Bruce *et al*, 1997; Hill *et al*, 1997) therefore implies that large number of UK population may have been exposed to the BSE agent and could potentially develop prion disease.

A total of 172 definite/probable cases of vCJD deaths have been reported in the UK since 1990, out of the 1587 documented CJD related deaths (NCJDRSU, 2011). However, UK national vCJD prevalence study estimated (95 % confidence interval) that between 49 and 692 people per million of UK population (Hilton *et al*, 2004) or 109 people per million of UK population (de Marco *et al*, 2010) may be asymptomatic vCJD carriers. These data invariably suggest a significant impact on public health as a direct consequence of BSE contamination through consumption of infected cattle (SEAC, 2010; Belay and Schonberger, 2005), or transfusion of blood received from infected individuals (Wroe *et al*, 2006; HPA Press Office, 2009; NCJDRSU, 2011), or treatment with blood product received from infected individuals (Peden *et al*, 2010) or iatrogenic transmission through surgical instruments contaminated by infected individuals (Collins *et al*, 1999; Belay and Schonberger, 2005). Unfortunately, there are no reliable preclinical screening methods for vCJD, so that asymptomatic carriers may continue to donate blood and organs or undergo surgical intervention, thereby exposing more people to vCJD risk. Invasive surgical intervention potentially poses a higher risk of iatrogenic vCJD (Garske *et al*, 2006; Wadsworth *et al*, 2007) and CJD (WHO, 1999; Collins *et al*, 1999; de Pedro-Cuesta *et al*, 2011) transmission, for reasons which include direct exposure of tissues to the prion agent, and because intra-species transmission is generally more efficient.

1.5.2 Prions and the environment

Prions are emerging environmental contaminants (Snow *et al*, 2007). The possible routes of prion deposition in the environment includes sheddings of diseased animals (urine, faeces, saliva, blood), decomposing carcasses, carcasses disposed in landfills, composting of carcasses, wastewater from rendering plants and slaughter houses, effluent from hospital and research facilities and land spreading of biosolids (Mathiason *et al*, 2006; Kirchmayr *et*

al, 2006; Hinckley *et al*, 2008; Haley *et al*, 2009). For example, at the onset of the BSE crisis in the UK, an estimated 6000 suspected BSE infected carcasses were disposed off in 59 landfill sites (The Environment Agency, 1997; Saunders *et al*, 2008), which may pose potential prion contamination risks to the environment as current knowledge of prion behaviour indicates. Therefore, appropriate risk assessment to determine the fate of the prions in these environments may be required.

Prion in the environment poses potential health risks to man and animals. Ingestion of prion contaminated soil and vegetation during foraging constitutes potential TSE risk, especially in the light of oral transmissibility of scrapie and chronic wasting disease (CWD) (Hadlow *et al*, 1982; Miller *et al*, 2004; Sigurdson *et al*, 1999; O'Rourke *et al*, 1997), which highlights the risks of TSE transmission through ingestion of prion contaminated vegetation and soil-bound prion during foraging (Beyer *et al*, 1994; Johnson *et al*, 2007; Seidel *et al*, 2007). Therefore, prion contaminated environment poses agricultural challenges for farmed animals (sheep and deer) industry and sustainability challenges for free-ranging animals (deer and elk) population. There is also potential risk to humans exposed to such environment, or who may consume the meat of animals infected through such environment.

1.5.3 Resilience of the TSE agent and its implications

The TSE agent is resistant to common endogenous and environmental proteases, and withstands conventional physical and chemical sterilisation, inactivation and decontamination procedures (Taylor *et al*, 1994; Taylor *et al*, 1999; Kimberlin *et al*, 1983; Brown *et al*, 1982; McDonnell and Burke, 2003; Edgeworth *et al*, 2011). The TSE agent binds avidly to metal, glass and plastic surfaces (Zobeley *et al*, 1999; Flechsig *et al*, 2001; Weissmann *et al*, 2002; Yan *et al*, 2004). Autoclave sterilisation (moist heat-based inactivation) of prion contaminated metal have been reported to result to greater attachment and stabilisation of the prion agent making it refractory to inactivation (Flechsig *et al*, 2001; Zobeley *et al*, 1999; Walker *et al*, 2007; Fernie *et al*, 2007). These findings suggest that prion contaminated medical devices (surgical and dental instruments) processed by routine cleaning procedure may still retain prion infectivity and hence constitute potential route of vCJD and CJD transmission through reuse of such instruments hence prion contaminated surgical instruments remain a potential source of iatrogenic transmission (WHO, 1999), and for this reason, effective decontamination of reusable medical devices remain a crucial and desirable aspect of instrument management in modern surgical practice.

Prions deposited in the environment, for example in soil, remain highly stable and retain its infectivity and hence the environment is a stable reservoir of prion infectivity (Wiggens, 2008; Johnson *et al*, 2007; Saunders *et al*, 2009; Tamgüney *et al*, 2009; Maddison *et al*, 2010; Wilham *et al*, 2010). For example, scrapie prion interred in soil for three years retained about 50 % of its infectivity (Brown and Gajdusek, 1991). Furthermore, an epidemiological study of scrapie has shown persistence of the scrapie agent under environmental conditions for at least 16 years (Georgsson *et al*, 2006). The persistence of prions in the environment suggests that it is not readily degraded by environmental proteases.

Resilience of the scrapie agent may also account for the recurrence of prion infection in disease-free livestock re-introduced into environment that previously held scrapie contaminated flock (Georgsson *et al*, 2006). Persistence of soil-bound CWD agent in the environment have been implicated in the horizontal transmission of CWD resulting in the CWD problem plaguing both farmed and free-ranging deer and elk in endemic proportions in parts of Canada and the United States (Miller *et al*, 2004). Clearly, CWD poses significant public health and economic concerns for deer and elk farming, as well as a threat to both human and animal health similar to BSE and scrapie.

In the absence of reliable methods for detecting TSE in asymptomatic hosts, and as medical intervention for prion diseases remain elusive, control of TSE related risks and the development and improvement of decontamination practices remain a responsible option (Walker *et al*, 2008). Therefore, decontamination methods that are able to effectively destroy the prion agent at point sources including surgical and laboratory instruments, soil, farm implements, carcasses, and effluent from meat processing remain highly desirable.

1.6 Prion decontamination

Prion decontamination generally refers to a reduction in prion titre, or removal of prion infectivity or biological activity by processes such as detachment, destabilisation (inactivation) and degradation, which causes an irreversible alteration of the chemical structure of prions (Croud *et al*, 2008). Detachment and stabilisation may occur in the absence of prion degradation (Lemmer *et al*, 2004) which may account for loss of PrP^{Sc} while infectivity remains. However, the terms prion decontamination, inactivation and degradation are used interchangeably. The resilient nature of the TSE agent has meant that stringent decontamination methods are often required to destroy its infectivity.

In 1960, Pattison and Millson reported that scrapie prion was resistant to formalin and heat treatment. The reasons for this resilience nature was attributed to it proteinaceous nature

and the high β -sheet structural composition (Taylor, 1991; McKinley *et al*, 1991). The misfolded structure of prions is stabilised by disulphide bonds, so that physical and chemical processes which unfold (unfolding agents) and cleave disulphide bond (disulphide bond reductases) may invariably alter its chemical characteristics, resulting in the loss of prion infectivity. Physical processes that could unfold prions include: heat treatment, pH adjustment (<3 or >9), ultrasonic energy, infrared, electromechanical and microwave radiation, high pressure, electrical and magnetic fields, energetic vibration (magnetic or vortex stirring), electronic beam irradiation, and laser treatment], and the chemical processes that unfold prions include: treatments with organic solvents and solutes, chaotropic agents, chemical surfactants, inorganic salts, and agents that cleave disulphide bonds such as thioglycol.

1.6.1 Chemical and physical prion decontamination methods

The commonly reported prion decontamination methods includes chemical and physical treatments such as dry heat treatment, steam autoclaving and radio-frequency gas-plasma treatments, and treatments with acidic SDS, formic acid, peracetic acid, 2 N sodium hydroxide, and sodium hypochlorite solution (bleach) containing 20,000 ppm active chlorine and combination of these components (Ernest and Race, 1993; Taylor, 1999; Lemmer *et al*, 2004; Baxter *et al*, 2005, Peretz *et al*, 2006). The World Health Organisation (WHO) recommendation for prion decontamination include porous load autoclaving at 134 °C for 18 min and immersing contaminated material in 1 M NaOH and/or 20,000 ppm NaOCl for 1 h at 20 °C (WHO, 1999). Similar chemical and physical treatment methods are recommended by the Society of Healthcare Epidemiology of America (SHEA) in its guideline for disinfection and sterilisation of prion-contaminated medical devices (Rutala and Weber, 2010). The methods considered to be most reliable for effective prion destruction are incineration (Defra, 2005; Jennette, 2002), thermal hydrolysis (Somerville *et al*, 2009) and alkaline hydrolysis (Thacker and Kastner, 2004; Kalambura *et al*, 2005; Yokoyama *et al*, 2006).

1.6.1.1 Limitations of physical and chemical decontamination methods

The chemical and physical prion decontamination methods are harsh, potentially hazardous and destructive, and hence are unsuitable for important applications such as decontamination of delicate medical devices, and recoverable animal by-products (e.g. specified risk materials [SRM]) and carcasses. For example, the WHO recommended protocol for prion decontamination has been shown to corrode and blacken steel surfaces of medical instruments causing irreversible damaging effects (Fig. 1.6) (Brown *et al*, 2005).

High temperature (134 °C) autoclaving is the UK hospital standard for decontamination of surgical instruments, the effectiveness of which remains questionable (Taylor, 1999; McDonnell and Burke, 2003; Edgeworth *et al*, 2011). For example, surgical instruments decontaminated by the routine hospital process were found to retain significant amounts of protein (Murdoch *et al*, 2006), and prion is particularly known to adhere to steel surfaces. The spongiform encephalopathy advisory committee (SEAC) in its guideline for assessing and comparing the efficacy of decontamination methods recommends that an effective decontamination method requires to achieve at least a five-log reduction in infectivity (SEAC, 2006). Such a method would also need to achieve at least one-log reduction in infectivity greater than the ID₅₀. The standard autoclaving procedure (121 °C, 15 min) has been reported to achieve between two- and three-log reductions in infectivity. A similar level was reported for rodent-adapted human prion treated with 1 M NaOH for 1 h or autoclaving at 134 °C for 18 min (Lawson *et al*, 2007). The use of higher sterilisation temperature over extended incubation time (up to 30 min), such as those used in the UK hospital instrument sterilisation has been reported to achieve a four-log reduction (Kimberlin *et al*, 1983; Taguchi *et al*, 1991) which suggests that current UK hospital sterilisation standard falls short of the desirable standard and hence reuse of contaminated medical devices sterilised by the existing method could constitute healthcare risks. Prion decontamination with chlorine at concentrations up to 5000 ppm for 30 min also achieved a four-log reduction in infectivity (Brown *et al*, 1982). Only very high chlorine concentrations up to 10,000 ppm achieved more than four-log reduction in infectivity (Rutala and Weber, 2001). These reports suggest that most of the current chemical and physical prion decontamination methods such as treatment with NaOH, NaOCl and autoclaving may not be effective.



Fig. 1.7: Mayo–Hager carbide-faced needle holders showing severe corrosion and blackening after immersion in bleach for 1 h (The instrument on the top, labeled 3), and autoclaving five times in 1N NaOH (The instrument below, labeled T) respectively (Brown *et al*, 2005).

The use of huge amounts of chemical agents for prion decontamination, especially in large scales application constitutes potential health hazards relating to spillage, and emission of noxious and irritating gases. Another problem is the handling and disposal of high levels of chemical contaminated effluents and hydrolysates which may end up in the environment (Sehulster, 2004; Greenlee *et al*, 2008). Furthermore, incompletely destroyed prion released into the environment could potentially constitute a pathway for prion contamination.

The decontamination of prion infected tissues by alkaline hydrolysis (Kalambura *et al*, 2005; Yokoyama *et al*, 2006) results in by-products that are extremely degraded and have high residual salt concentration, therefore retaining little commercial value (Coll *et al*, 2007). For these reasons, alkaline hydrolysis is unsuitable for application in the rendering of recoverable SRM and carcasses which, for example, is an estimated 17 million tonnes in the EU alone (Taylor and Woodgate, 2003). Therefore, rendering processes that effectively destroy prion infectivity but allows recovery of full value of recoverable protein-rich products is economically and environmentally valuable and desirable.

In the UK and Canada, the approved method for disposal of TSE infected carcasses is incineration (UK animal by-products regulations 2005; Canadian Food Inspection Agency, 2006). Incineration is considered to be most effective method for prion destruction in non-recoverable materials, although some reports suggests that it may not be completely effective for the destruction of the prion agent (Brown *et al*, 1990; Brown *et al*, 2004; Woodward, 2004). Incineration is impractical for the decontamination of recoverable and reusable materials (e.g. animal by-products), surgical and laboratory instruments, soil-bound prion, vegetation and farm infrastructure (Saunders *et al*, 2008). Incineration is also not a practical solution during large outbreak of BSE, scrapie or CWD requiring mass culling. In addition to environmental impact, and regulatory considerations, capacity limitation and the operational cost of running such facilities is high.

In general, it could be argued that chemical and physical prion decontamination methods are harsh, potentially hazardous, environmentally polluting, and inappropriate for use in decontamination of delicate and recoverable materials, and may not be highly effective for complete destruction of prion infectivity. In addition, prion contaminated environments cannot be easily and practicably remediated by chemical and physical prion decontamination procedures. Therefore, appropriate remediation measures are required to decontaminate prion contaminated environment and materials without the limitations associated with most physical and chemical methods. In this vein, enzymatic (microbial)

degradation appears to be the most plausible approach (Huang *et al*, 2007; Rapp *et al*, 2006; Saunders *et al*, 2011). This method while potentially able to destroy prion infectivity is less likely to destroy the decontaminated material, and it is also the most practicable approach for application in the remediation of prion contaminated environment without destroying the environmental biota and/or ecology.

1.6.2 Enzymatic degradation of prions

The enzymatic inactivation of prions was listed as one of the science objectives and targets in the UK Transmissible Spongiform Encephalopathy Directorate (2003-2006) Science Strategy (Defra, 2003). The Alberta Prion Research Institute (APRI) Canada, who co-funded this work with Middlesex University, London, UK is also committed to the prospects of enzymatic prion inactivation as an integral part of its overall prion research strategy for the inactivation of prion in the environment.

The earliest reports of enzymatic degradation of the prion were the inactivation of the scrapie agent by the protease, pronase (Cho, 1983), and the degradation of the BSE agent by a combined heat and enzymatic treatment, in which BSE infected brain homogenate was preheated at 115 °C for 40 min followed by digestion with keratinase of *Bacillus licheniformis* PWD-1 for 4 h (Shih, 2002). Subsequently, the degradation of prions by enzymatic methods and methods that combine other treatment (physical and chemical) methods have been widely reported (Hui *et al*, 2004; McLeod *et al*, 2004; Jackson *et al*, 2005; Lawson *et al*, 2007; Yoshioka *et al*, 2007; Croud *et al*, 2008, Mitsuiki *et al*, 2010).

Similar to the method reported by Shih (2002), BSE prion infected brain homogenate was digested for 1 h with *Bacillus licheniformis* PWD-1 keratinase at 50 °C after heat pre-treatment for 40 min at 115 °C (pressure, 75 KPa) (Langeveld *et al*, 2003). In this experiment, the digestion time was reduced to 1 h and strong chemical detergent (N-laurylsarcosine) was included in the heat pre-treatment step. Relatively weaker detergents such as Triton X-100 and Sodium deoxycholate resulted in reduced digestion efficiency, highlighting the important role of detergents in enzymatic prion degradation mechanisms. Although undetectable levels of PrP^{Sc} was achieved under these conditions as determined by western blot analysis, no test of residual infectivity were reported to verify the efficacy of this method.

Enzymatic decontamination of MBM containing amyloid prion protein by the proteases of anaerobic thermophilic prokaryotes (*Thermoanaerobacter* S290, *Thermosipho* VC15, and *Thermococcus* VC13) and soil mesophilic bacterium (*Streptomyces* subsp. S6) was reported by Tsirolnikov *et al* (2004). The supernatants of *Thermoanaerobacter* subsp.

S290 and *Streptomyces* subsp. S6 effectively and rapidly hydrolysed mouse-adapted BSE (6PB1) PrPres resulting in a significant 85 % and 88 % loss of PrP signal intensity after digestion at 60 °C and 30 °C respectively for 24 h. Although all the reported strains S290, S6, VC13 and VC15 hydrolysed feather keratin, VC13 and VC15 showed weak activity towards mouse-adapted 6PB1 BSE strain at low incubation temperatures, but showed higher activity towards MBM protein at 80 °C compared to S290 and S6 (Tsiroulnikov *et al*, 2004). The weak activity by VC13 and VC15 at lower temperature was attributed to protease inhibition, protease inactivation by small brain molecules or by detergents resulting from the purification of PrPres. Also, the protease, Versazyme™, was reported to rapidly diffuse into bone and soft tissue particles of MBM in 30 min and 4 h respectively and hence able to potentially solubilise and inactivate the prion agent (Coll *et al*, 2007). The prospects of safe and efficient decontamination of MBM and SRM imply the recovery of huge amounts of useful and economically viable resources which would normally end up in incinerators and landfills.

BSE-passaged mouse brain homogenate digested with the thermostable protease, properase, at 65 °C for 30 min and pH 12, resulted in loss of prion infectivity and a slight increase in disease incubation time in a bioassay from 120 ± 8.5 days for untreated sample to 147.5 ± 7.71 days for the enzyme treated sample (McLeod *et al*, 2004). Prior to intracerebral inoculation, the digested sample was further heated to 100 °C in order to inactivate the enzyme. Such high temperature treatment on its own could significantly denature prions, reduce infectivity and cause loss of PrP^{Sc} immunoreactivity (Somerville *et al*, 2002; Taylor *et al*, 2002). However, the extent to which the reported loss of prion infectivity is attributable to protease inactivating temperature was not defined.

An enzyme-detergent degradation method which combined proteinase K and pronase in the presence of SDS has been reported to completely destroy infectivity of metal-bound vCJD-infected brain homogenate (Jackson *et al*, 2005). The method required heat pre-treatment of the prion infected material in 2 % SDS at 100 °C for 15 min, and then cooled to room temperature followed by sequential treatment with PK and pronase at 30 min intervals. An alteration of the treatment sequence, for instance, treating the digest with pronase before PK resulted in reduced degradation efficiency. Similarly, an enzyme-detergent degradation method in which rodent-adapted human prion was digested at 50- 60 °C for 30 min, followed by autoclaving of the digest has been described (Lawson *et al*, 2007). Furthermore, a prion degrading composition comprising an effective amounts of an oxidizing agent, surfactant and one or more proteases was reported to completely destroy prion when digested at ≥ 50 °C for ≥ 20 min (Croud *et al*, 2008). In the described methods,

sequential application of the composite degradation agents was necessary in order to achieve optimal synergistic effect, and to prevent inhibition of protease activity. However, such sequential, multi-step procedure for enzymatic decontamination of prion is complex and impractical for commercial application (Edgeworth *et al*, 2009).

Protease of *Bacillus licheniformis* MSK-103 was reported to inactivate sheep, mouse and hamster prion brain homogenate (1 %) in 20 h at 37 °C in the absence of heat pre-treatment or detergent (Miwa *et al*, 2006). This protease (optimum pH 9-10 and optimum temperature 60-70 °C) was reported to degrade prion more effectively than *Bacillus licheniformis* PWD-1 keratinase. The protease of *Bacillus Thermoproteolyticus* Rokko was also reported to sufficiently degrade prion, but only in the presence of SDS, a neutral salt (required to activate the enzyme) and a metal ion (Miwa *et al*, 2006). In another study, the protease of *Bacillus licheniformis* MSK-103 exhibited high degradation activity against scrapie (Sc237 brain homogenate) and BSE prions, degrading them to undetectable levels at 37 °C in 1 h and 50 °C in 20 h respectively, at pH 9-10, without pre-treatment of the prion sample or addition of detergent (Yoshioka *et al*, 2007). However, the high pH digestion conditions may have resulted in alkaline hydrolysis of the prion substrate. In a follow-up study, a combined treatment of MSK-103 protease and SDS at 50 °C for 20 h reduced infectivity of the scrapie brain homogenate, resulting in a survival rate of 40 % in bioassay in which the digest was inoculated in Tg52NSE mice, and experiment terminated at 600 days (Yoshioka *et al* 2007). Also, this protease was reported to degrade scrapie prion agent bound to plastic surface more effectively than both PWD-1 keratinase and PK (Yoshioka *et al*, 2007). Alkaline proteases E77 from *Streptomyces* sp (Hui *et al*, 2004) and NAPase from *Nocardiopsis* sp TOA-1 (Mitsuiki *et al*, 2006) were also reported to degrade scrapie prion within 3 min at 60 °C and pH >10 without any further chemical and physical treatments. In addition, six proteolytic microorganisms isolated from cheese were identified to degrade scrapie-infected hamster (strain 263K) in 24 h at 30 °C and pH 8 (Muller-Hellwig *et al*, 2006).

Pilon *et al* (2009) reported that mouse-adapted scrapie prion brain homogenate digested with serine protease subtilisin 309 (from *Bacillus clausii*) at 55 °C under mild alkaline condition (pH 7.9) for 14 h, and inoculated in a mouse bioassay, resulted in a survival rate of 52 % in the challenged animals (C57Bl/6 mice), with mean incubation periods of 213 ± 0 days for untreated (positive control) samples and 248 ± 8 days for treated samples. Clearly, some attenuation of the prion infectivity resulted in extended incubation time in the experimental group. Although this result was described as statistically significant ($P < 0.001$) compared to control, the authors suggest that greater enzymatic degradation

efficiency could be achieved at higher pH (>9) and temperature conditions, and increased enzyme concentration.

Recently, a *Bacillus lentus* subtilisin strain has been genetically modified to synthesise an alkaline protease, MC3, of high proteolytic activity. This protease was reported to inactivate BSE 301V infectious mouse brain homogenate to immunochemically undetectable levels at 60 °C, pH (≥ 12) for 30 min (Dickinson *et al*, 2009). VM mice inoculated with the MC3-digested material achieved a survival rate of 66.6 %, compared to proteinase K digested substrate, suggesting that a considerable reduction in prion infectivity was achieved.

Destruction of prion infectivity by enzymatic digestion suggests that composting could be an important method for disposal of prion contaminated carcasses. Composting of scrapie infected sheep has been reported by Huang *et al* (2007) in which scrapie infected tissue was buried in compost piles. The compost temperature reached 60 °C and remained so for two weeks before gradually declining to ambient temperature. After 180 days, the tissue weight reduced and PrP^{Sc} was degraded to undetectable levels, clearly indicating significant microbial activity. Furthermore, degradation of scrapie prion in 90 days at a sustained temperature of 60 °C has recently been reported (Huang *et al*, 2010). Triantis *et al* (2007) also reported the composting of CWD-infected elk and deer brain homogenate at 55 °C resulting in partial or complete loss of PrP^{Sc} as determined by Western blot analysis. In these studies, no tests of residual infectivity were conducted to validate the efficacy of this method. However, these results suggest that proteolytic microorganisms that thrive at composting temperature range may be important for effective degradation of scrapie prions under composting conditions. Therefore, composting of prion infected carcasses could be highly important during disease outbreaks. Although composting is a potential method for disposing carcasses (Glanville *et al*, 1997) and is an approved method in Canada for disposal of TSE infected carcasses (Canadian Food Inspection Agency, 2006), large scale composting may be problematic both in terms of practicality and efficiency and if not fully degraded may constitute a potential contaminant to the local environment.

In summary, most of the reported enzymatic prion degradation methods require that the prion substrate be rendered proteolytically susceptible by high temperatures heat pretreatment, addition of chemical surfactant/detergent, denaturants and/or oxidizing agents, incubation in high alkaline condition, digestion for long period of time, or by combining of some of these conditions (Langeveld *et al*, 2003; McLeod *et al*, 2004; Jackson *et al*, 2005; Croud *et al*, 2008). These conditions are harsh, environmentally and

economically unsustainable and limit potential practical application of the enzymatic prion degradation method (Pilon *et al*, 2009). Therefore, an enzymatic degradation method that combines moderate pH and temperature conditions, short digestion time, and degradable, non-toxic biological detergents will be potentially useful and highly desirable. Such a method has to be simple, practical, safe, cost effective, environmentally friendly and commercially plausible.

1.6.2.1 Mechanism of enzymatic degradation of prion

Enzymatic degradation of prions generally involves unfolding of the β -sheet structure of PrP^{Sc} and breaking of disulphide and hydrogen bonds (Langeveld *et al*, 2003). Therefore, processes that unfold proteins, promote disruption of hydrogen and disulphide bonds and enhance solubilisation would potentially facilitate enzymatic degradation of prions. Such processes including heat denaturation, acidic or alkaline hydrolysis, and solubilisation by detergents, singly or synergistically increase proteolytic access into the prion matrix and hence greater proteolytic attack (Dickinson *et al*, 2009; Lawson *et al*, 2007; Oesch *et al*, 1994; McLeod *et al*, 2004, Bolton *et al*, 1984). It could be argued however that the underlying mechanistic pathway of enzymatic prion degradation may vary according to the specific enzymes, prion strains and digestion processes involved.

1.6.2.2 Factors that affect enzymatic degradation

The enzymatic degradation of prions is affected by factors such as variability of PrP^{Sc} susceptibility to proteolytic attack (Fernie *et al*, 2007), state and matrix of prion material (whether they are intact tissues, fat laden particles or homogenate) (Murayama *et al*, 2006; Coll *et al*, 2007), rate of enzymatic activity and digestion conditions (Fichet *et al*, 2004; Fichet *et al*, 2007; Giles *et al*, 2008). Enzymatic activity, for instance, could be reduced or lost by heat denaturation, presence of excess surfactants, and enzyme autolysis. Therefore, an effective and desirable enzymatic degradation method has to be robust and useful for various prion decontamination applications.

1.7 Biosurfactants

Biosurfactants or microbial surfactants are extracellular or membrane-associated amphiphilic surface-active biomolecules produced by a variety of microorganisms such as bacteria, yeast and fungi (Rosenberg, 1986; Hommel, 1990; Muthusamy *et al*, 2008). *Pseudomonas aeruginosa* for example is a prolific producer of biosurfactants (Edward and Hayashi, 1965; Hisatsuka *et al*, 1971; Soberon-Chavez *et al*, 2005). Biosurfactants are classified into glycolipids, lipopeptides, phospholipids, polymeric and particulate forms depending on their chemical composition and structure (Rosenberg and Ron, 1999).

Generally, biosurfactants are composed of a hydrophilic (head) and hydrophobic (tail) moieties (Desai and Banat, 1997; Nitschke and Coast, 2007). The hydrophilic moiety is usually a carbohydrate, amino acid, phosphate, cyclic peptide, carboxylic acid, or alcohol, and the hydrophobic moiety is mostly a long-chain fatty acid, or fatty acid derivatives such as hydroxyl fatty acid or α -alkyl- β -hydroxy fatty acid. The complete structure of rhamnolipid, a glycolipid biosurfactant of *Pseudomonas aeruginosa* (Fig. 1.7) was described by Edward and Hayashi (1965).

Biosurfactants are characteristically able to reduce viscosity, lower surface and interfacial tension, and also able to stimulate desorption rate, substrate dispersion, flocculation, cell aggregation and emulsification (Karanth *et al*, 2005; Volkering *et al*, 1998; Haferburg *et al*, 1986). These properties have been explored for various industrial applications.

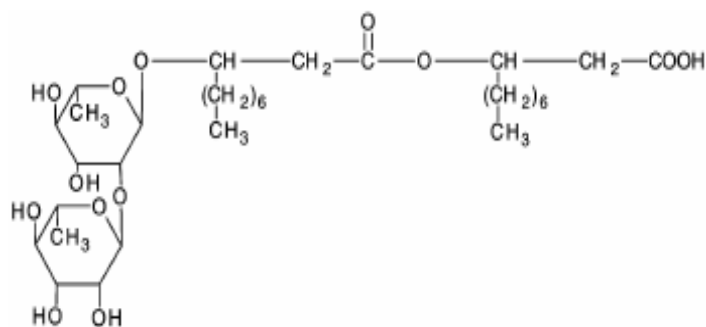


Fig. 1.8: Structure of rhamnolipid biosurfactant from *Pseudomonas aeruginosa* consisting of two rhamnose subunits that are linked to two β -hydroxydecanoic acid in a side chain.

In the petroleum industry, biosurfactants have application for enhanced oil recovery from reservoirs, transportation and pipelining of oil and cleaning of oil barges and tanks due to their ability to reduce viscosity of heavy oil (Desai and Banat, 1997; Wang *et al*, 2007). Their ability to increase the surface area of recalcitrant hydrophobic compounds such as hydrocarbons and pesticides in soil and water environments enable microbial access and enhanced degradation of such compounds, hence it is useful for environmental bioremediation applications (Noordman and Jassen, 2002; Ochsner and Reiser, 1995; Deziel *et al*, 1996; Kosaric, 2001; Okoroma, 2006). The anti-microbial, anti-viral, anti-tumor, and anti-adhesive activities of biosurfactants are also becoming increasingly important (Cao *et al*, 2009; Benincasa *et al*, 2004; Soberon-Chavez *et al*, 2005; Brzozowski *et al*, 2011). For example, the anti-adhesive property is useful for inhibiting bacterial adherence to steel surfaces and reducing formation of biofilms (Bagge *et al*, 2001; Muthusamy *et al*, 2008; Brzozowski *et al*, 2011). They are therefore used to impregnate medical equipment surfaces to reduce adherence of pathogen and formation of biofilms

(Velraeds *et al*, 1997; Rodrigues *et al*, 2006). Biosurfactants are also used in the agricultural industry for biocontrol and in the food processing industry as emulsification and de-emulsification agents. They have also found important applications in cosmetic, pharmaceutical and therapeutic products (Nitschke and Coast, 2007; Singh *et al*, 2007; Muthusamy *et al* 2008).

Biosurfactants are a preferred environmentally friendly alternative to chemical surfactants because of their high detergency, high surface and interfacial activity, low toxicity, unique structure and temperature and pH tolerance (Muthusamy *et al*, 2008). For example, biosurfactants of *Pseudomonas aeruginosa* have been reported to lower surface tension of water from 72 mN/m to 25 mN/m (Hisatsuka *et al*, 1971); whereas the chemical surfactant SDS was only able to lower surface tension to 37 mN/m. In general, biosurfactants are more effective and efficient than chemical surfactants, and their critical micelle concentration (CMC) is about 10-40 times lower, hence less biosurfactant is required to achieve a maximum decrease in surface tension (Desai and Banat, 1997).

Currently, there is no reported use of biosurfactant in prion degradation. However, there has been varying reports of the effects of chemical surfactants/detergents on prion infectivity. For example, it has been reported that surfactant/detergent increased apparent titre of PrP^{Sc} preparation (Somerville and Carp, 1983) and increased resistance to prion inactivation during steam sterilization (Yan *et al*, 2004), whereas Tixador *et al* (2010) reported that detergents did not alter infectivity estimates when used to solubilise prion. However, the important role of surfactants/detergents in enzymatic prion degradation has been severally reported (Jackson *et al*, 2005; Lawson *et al*, 2007; Yoshioka *et al*, 2007). Considering the biochemical properties of biosurfactants and their advantages over chemical surfactants, they are potentially important alternative for use in the enzymatic degradation of prions.

1.8 Research hypotheses for this study

Infectious prion protein (PrP^{Sc}) is structurally similar to feather keratin in terms of its β -sheet content (Shih, 2002; Langeveld *et al*, 2003; Tsirolnikov *et al*, 2004). Feathers consist of approximately 90 % keratin protein (Fraser *et al*, 1972), and its β -sheet conformation confers it with mechanical strength and stability, and resistance to degradation by common proteases. Despite the relative abundance of feathers in nature, they do not persist in the environment suggesting their removal by some naturally occurring processes which have been attributed to feather degrading microorganisms (Noval and Nickerson, 1959). It is therefore hypothesised that owing to the structural

resemblance of infectious prion agent (PrP^{Sc}) and feather keratin, feather degrading microorganisms, specifically their specially expressed enzymes, are potentially able to hydrolyse feather keratin and prion substrates. It is further hypothesised that the ability of feather degrading microorganisms to grow and hydrolyse feather keratin substrate could be used as a basis for screening feather degrading microorganisms and evaluation of their proteolytic enzymes.

1.9 Aim and Objectives

The aim of this research was to investigate the potential of a novel bacterial keratinase to degrade pathogenic prion protein, specifically, ME7 scrapie infected brain homogenate.

The specific objectives of this research were to:

1. isolate, identify and characterise proteolytic microorganisms of significant keratinolytic activity from primary effluent and farmyard wastes.
2. optimise process for the production, purification, characterisation and evaluation of keratinase.
3. investigate the effectiveness of the produced keratinase in the *in vitro* degradation of melanised feather keratin and ME7 scrapie prion, and to optimise the degradation conditions.
4. conduct *ex vivo* scrapie cell assay and *in vivo* mouse bioassay to evaluate residual infectivity of keratinase-digested scrapie prion, and to determine the efficacy of this enzymatic treatment for degradation of scrapie prion.

Chapter 2: Materials and methods

2.1 Experimental approach

In order to find keratinases of prion degrading potential, an experimental approach was designed which included the isolation and characterisation of proteolytic bacteria and their keratinases, investigation of keratinase's ability to degrade feather keratin and scrapie prion, optimisation of the degradation method and evaluation of residual infectivity by cell culture assay and mouse bioassay. This chapter is divided into three main experimental stages: isolation and characterisation of proteolytic microorganisms, keratinase and biosurfactant, degradation of keratin substrates and scrapie prion by keratinase, and residual infectivity evaluation of keratinase-digested scrapie prions. An overview of the experimental approach is presented in Fig. 2.1, with each experimental stage distinguished by different colour fills. All materials used in the experiments are described in the procedure for individual experiments.

2.2 Isolation and characterisation of proteolytic microorganisms, keratinase and biosurfactant

2.2.1 Isolation of microbial strains

Farmyard waste (Tork *et al*, 2010) and sewage sludge (Drouin *et al*, 2008; Abdul-Hafez and El-Sharoumy, 1990) are important sources of microbial population and extracellular proteases, and feather degrading bacteria utilise feather meal as growth substrate (Brandelli and Riffel, 2005). To isolate potential feather degrading bacteria, environmental samples were collected from primary effluent (Deephams Sewage Treatment Facility, Thames Water, Edmonton, North London, UK) and poultry/animal wastes (A.K Woods Poultry Farm, Fold Farm Partners and Leamon Pig Farm Ltd, UK). The samples were diluted and plated out in feather meal agar (FMA) plates composed of agar and 10 % commercial feather meal (Chettles Ltd, UK) made up in Minimum Growth Medium (MGM). The Minimum Growth Medium (phosphate basal solution) was composed of (in g/l): NaCl, 0.5; KH₂PO₄, 0.7; K₂HPO₄, 1.4; MgSO₄·7H₂O, 0.1; pH 7 (Wang and Shih, 1999). The plates were incubated at 30 °C, 37 °C and 50 °C in a Binder incubator (Binder, USA) and sampled at 24, 48 and 72 h. Single colonies were passaged twice on FMA plates to obtain purified microbial strains. The purified isolates were cultured in nutrient broth and stored in 0.5 ml aliquots in liquid nitrogen at - 196 °C.

Flowchart of experimental approach

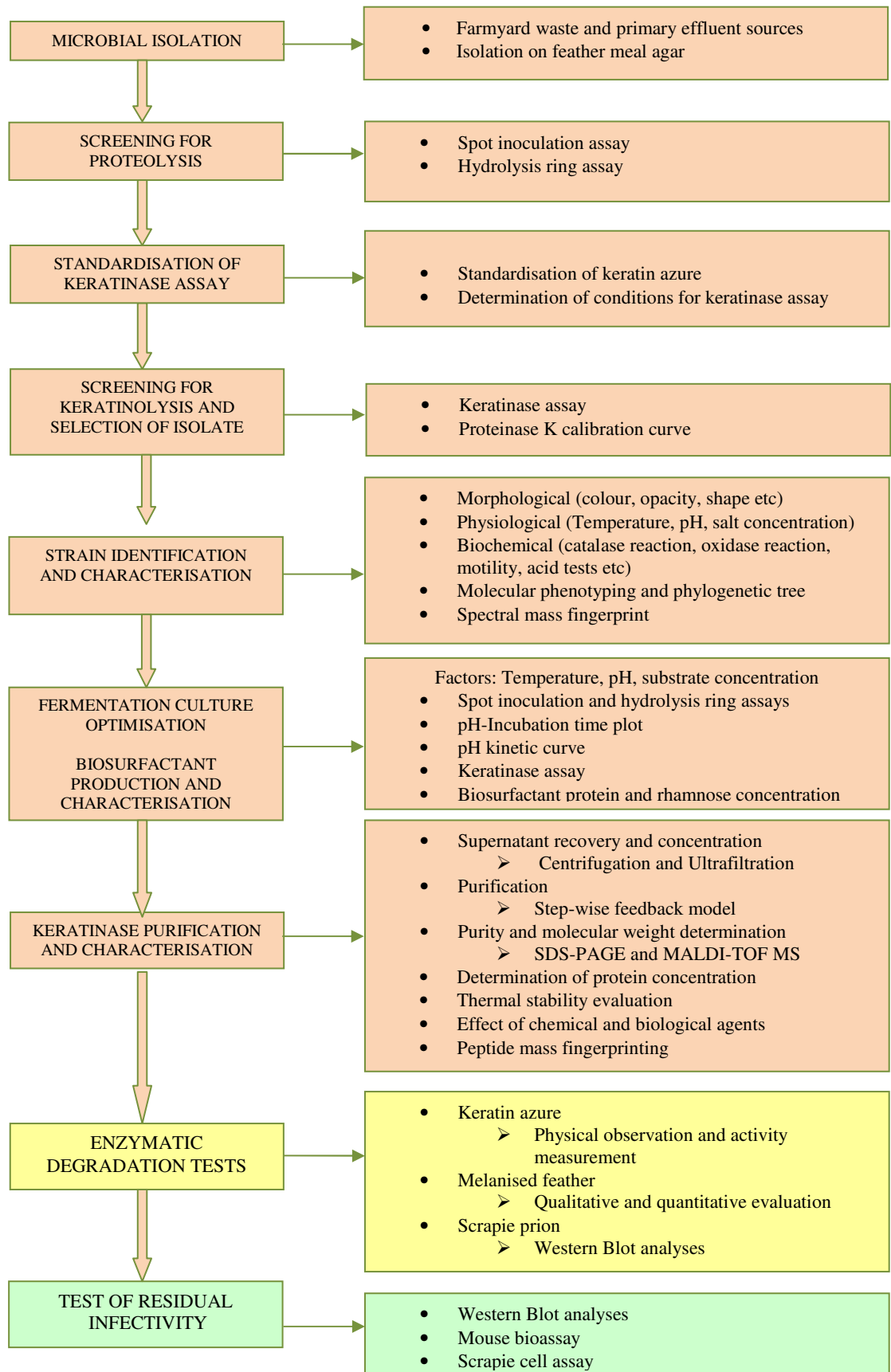


Fig. 2.1: Flowchart of experimental approach detailing experimental stages from microbial isolation to application of enzymatic product (keratinase) in degradation of ME7 scrapie prion strain.

2.2.2 Fermentation culture and crude enzyme extraction

Important biomolecules such as proteases are synthesised in fermentation cultures by the metabolic conversion of growth substrates. Crude enzyme extract is then obtained by centrifugation and filtration to remove microbial cells and other debris. MGM (10 ml) supplemented with 1.1 % commercial feather meal was autoclaved and inoculated with a loopful (approximately 20 µl) of single colony grown on nutrient agar plate (preculture) and incubated at 250 rpm for 16 h at the isolation temperature. One ml of the preculture was inoculated into 50 ml of growth medium (main culture) and incubated for 24 h at pH 8. Crude enzyme extract was recovered by centrifugation of the culture medium at 4750 g for 20 min in Rotina 420R centrifuge (Hettich, Germany) and the supernatant collected and ultra-filtered under vacuum through the 0.45 µm sterile membrane (Pall Corporation, USA). *Bacillus licheniformis* PWD-1 (ATCC 53757) was also grown in MGM supplemented with 0.5 % feather meal at 50 °C for 24 h (Wang and Shih, 1999) and crude keratinase was extracted as described above. The microbial cells and the crude enzyme extracts were used to screen for proteolysis.

2.2.3 Screening for proteolytic microorganisms

2.2.3.1 Spot inoculation assay

The spot inoculation assay is a casein-agar method for detecting proteolytic bacteria by their ability to hydrolyse and utilise casein as growth substrate. Screening for proteolytic microbial isolates was conducted by the spot inoculation assay and evaluated according to cell growth and hydrolysis of casein substrate. The casein-agar plates were prepared as 1% (w/v) each of casein (Fisher Scientific, UK) and agar (Oxoid, UK) mixed in sterile deionised water. The mixture was autoclaved at 121 °C and 10.26 bar) for 15 min in Priorclave autoclave (Priorclave, UK), allowed to cool to 50 °C and dispensed into plates. One loopful (approximately 20 µl) of a single bacterial colony was inoculated at a spot onto casein-agar plates and incubated for 24 h at 37 °C and 50 °C. Similarly, screening for proteolysis was demonstrated with some known microbial strains: *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418, *Staphylococcus aureus* NCTC 6571, *Bacillus subtilis* NCTC 10400 and *Bacillus stearothermophilus* NCTC 10007 (Health Protection Agency, UK).

2.2.3.2 Hydrolysis ring assay

The hydrolysis ring assay is a casein-agar based method that is useful for characterising crude enzyme extract by the extent of a “hydrolysis ring”. To determine proteolytic activity of crude enzyme extracts, wells were aseptically made in casein-agar plates using a sterile 7 mm diameter puncher. The wells were inoculated with 100 µl of crude culture extract

and incubated at 30 °C, 37 °C, and 50 °C for 24 h and the hydrolysis ring indicating proteolytic activity were compared. Validation experiment for hydrolysis ring assays was performed at 1:0; 1:10; 1:100; and 1:1000 dilutions of crude extract incubated at 50 °C for 24 h, and the hydrolysis ring diameters were measured to compare proteolytic activity.

2.2.4 Standardisation of keratin azure substrate

Keratin azure is a suitable substrate for evaluating keratinolysis. However, commercial keratin azure (Sigma-Aldrich, UK) has been found to be heterogeneous resulting in variability in activity measurement. Standardisation of the substrate is therefore required to enable consistent and reliable keratinase activity measurement within and between samples. Keratin azure was incubated with 5 times the volume of distilled water it displaces for 1 h at 50 °C and 250 rpm in a shaking incubator (Stuart, UK). The reaction mixture was then centrifuged at 4750 g for 10 min and the absorbance (A_{595}) of the supernatant measured with Helios Epsilon Spectrophotometer (ThermoFisher, UK). The keratin azure was further washed with distilled water by shaking vigorously for 1 min. The procedure was repeated until the absorbance readings of the wash solution remained constant. The treatment effluent was drained and keratin azure dried in an oven overnight at 30 °C. Difference in the treated and untreated keratin azure was determined using the Mann-Whitney Test.

2.2.5 Screening for keratinolysis and selection of working isolate

2.2.5.1 Keratinase assay

Keratinase assay is a specific and quantitative test for measuring keratinase activity, enabling definitive comparison of different keratinases. To measure keratinase activity, treated keratin azure [0.4 % (w/v)] was added to 0.8 ml of 10 mM Tris HCl (pH 8.5) and 0.2 ml crude keratinase extract added and the reaction mixture was incubated at 50 °C for 1 h in a rotary shaker at 250 rpm. The reaction was stopped with 0.2 ml of 10 % (v/v) Trichloroacetic acid (TCA) and the mixture was centrifuged at 9300 g for 20 min in an Eppendorf S1500 refrigerated microcentrifuge (Eppendorf, Germany). The absorbance of the supernatant was measured at A_{595} for keratinase activity. One unit of keratinase activity (U) was defined as the amount of protease producing an absorbance change of 0.01 units (A_{595}). The control experiments were (a) reaction mixture without crude keratinase (enzyme activity control) and (b) reaction mixture without keratin azure substrate (crude extract absorbance control).

2.2.5.2 *Proteinase K calibration curve*

Proteinase K (PK) is an important enzyme for the degradation and characterisation of prion protein. The PK calibration curve relates PK and keratinase by their activity on keratin azure substrate, and hence useful for establishing a baseline for identifying potential prion protein degrading keratinases. PK activity on keratin azure substrate was measured for PK concentrations in the range of 0-1 mg/ml, and calibration curve constructed as activity (U/ml) against PK concentration (mg/ml).

2.2.6 *Microbial identification and characterisation*

2.2.6.1 *Morphological, physiological and biochemical tests*

2.2.6.1.1 *Colony morphology*

Morphological characteristics of bacteria colony such as shape, colour, opacity, size, shape, elevation, edge and smell are used to identify and classify bacteria. To determine colonial morphology, cell culture of the selected strain was incubated for 24 h and then serially diluted and plated on nutrient agar plates to produce single colonies. The colonial morphology was then observed and described.

2.2.6.1.2 *Gram staining*

Gram staining is used for classifying bacteria on the basis of their forms and cellular morphologies. Gram stain segregates all bacteria into two large groups; those that retain the purple colour of the primary stain (crystal violet) on the cell wall are described as Gram-positive and those that retain the pink color of the counterstain (safrinin) are Gram-negative. To carry out Gram staining, a small amount of culture was added to a drop of water on the slide and spread to an even thin film with an inoculation loop, and then fixed on the slide by air-drying and then over a gentle flame. The slide was flooded with crystal violet for 1 min, rinsed with water, and flooded with iodine (iodine is a mordant that binds the crystal violet to peptidoglycan layer of gram-positive cell wall) and rinsed again with water after 1 min. The slide was then flooded with alcohol (a decolourizer that removes stain from the gram-negative cells), rinsed with water after 10 seconds (leaving the decolourizer too long may remove stain from the Gram-positive cells as well), flooded with safrinin and rinsed with water after 1 min. The slide was dried and viewed under oil immersion (1000 × TM) with a bright-field compound microscope (Meiji, UK).

2.2.6.1.3 *Endospore test*

Endospores are small and typically oval-shaped dormant cells which are formed by sporulation of stressed cells due to extreme environmental conditions such as high temperatures and changes in pH, absence of growth nutrient and presence of inhibitory chemical substances. Endospores lack metabolism and reproduction but return to active

and productive (vegetative) state when favourable conditions return. Endospore test is used to identify bacteria of the genera *Bacillus* and *Clostridium* which are usually endospore forming, and generally appear green under the microscope while vegetative cells appear as pink.

To test for endospores, a strip of blotting paper was laid over a prepared slide (same as in 2.2.6.1.2) and then placed over a screened water bath. The blotting paper was saturated with the primary stain, malachite green and allowed to sit over the steaming water bath for 5 min. Stain was re-applied when it was beginning to dry out. The blotting paper was removed and the slide rinsed with water until the water ran clear. The slide was then flooded with the safrinin for 20 seconds and rinsed in water. The specimen was then viewed under oil immersion using a light microscope (magnification of 1000 × TM) (Meiji, UK).

2.2.6.1.4 Anaerobic growth

Anaerobic bacteria are those able to grow without oxygen or in the presence of other electron acceptors. It is an important characteristic for distinguishing and identifying bacteria. The anaerobic growth test was performed by streaking Nutrient Agar plate with cells from a fully grown colony and incubated at 50 °C for 48 h in an anaerobic vessel (Becton Dickinson, USA), and observed for cell growth. Absence of growth indicates that the organism is an aerobe and vice versa.

2.2.6.1.5 Motility test

Motility test is used to identify and characterise organisms according to the nature of their movement in a suspension or growth in a solid medium. Motile organisms such as *Escherichia Coli*, *Pseudomonas aeruginosa* and *Bacillus species* (except *Bacillus anthracis* and *Bacillus cerus*) move randomly throughout a liquid suspension when viewed under a microscope. Non-motile organisms such as *Staphylococcus*, *Streptococcus* or *Micrococcus* either move with Brownian motion or are motionless. In a solid medium, motile organisms form a diffuse growth zone around an inoculum stab whereas non-motile organisms form a single line growth along the original inoculum stab. To test for cell motility, cell suspension was prepared in a sterile tube with two drops (approximately 0.1 ml) of sterile distilled water. One drop was transferred onto a microscope slide and overlaid with a cover glass. The slide was examined under a light microscope (magnification 400×) (Meiji, UK).

2.2.6.1.6 *Oxidase test*

Oxidase test is used to screen for bacteria that produce the enzyme cytochrome oxidase C; one of the enzymes in the bacterium electron transport system. Oxidase enzyme activity indicates that the bacterium is an aerobe or facultative anaerobe. It is used to identify bacteria species which belong mainly to the *Enterobacteriaceae* and *Pseudomonas* genus. To test for oxidase reaction, Dryslide oxidase test kit (Difco, USA) was smeared with bacteria colony and observed for oxidase reaction after 2 min as recommended by the manufacturer. The test is said to be positive when a dark purple colour develops.

2.2.6.1.7 *Catalase test*

Catalase is the enzyme that breaks hydrogen peroxide (H_2O_2) into water and oxygen ($2\text{H}_2\text{O}_2 + 2\text{O}_2$). Bacteria are identified and characterised on the basis of catalase reaction. For catalase reaction, a microbial colony was smeared on a clean glass slide, and a drop of 3 % hydrogen peroxide was added. The formation of foam and release of bubbles from oxygen indicated a positive catalase reaction.

2.2.6.1.8 *Triple Sugar Iron Test (TSI)*

This is a combined test for detecting a bacterium ability to hydrolyse glucose, lactose and sucrose, and also for detecting the reduction of sulphur to hydrogen sulphide (H_2S). The TSI medium consists of lactose (1 %), sucrose (1 %) and small amount of glucose (0.1 %), as well as ferrous sulphate and pH indicator phenol red. To perform TSI test, the TSI slant was stab-inoculated followed by streaking the inocula across the slant surface and incubated at 50 °C for 24 h. A red/yellow (Slant/butt of tube) colour indicates glucose fermentation, and black precipitate in the medium indicates iron reduction.

2.2.6.1.9 *Nitrate to nitrite reduction*

The nitrate reduction test is used to detect reduction of nitrate (NO_3^-) to nitrite (NO_2^-). This is a common reaction of most bacteria present in soil. To test for nitrate reduction, nitrate broth was inoculated aseptically and incubated at 50 °C for 24 h. After the incubation, a dropful each of sulphanilic acid and α -naphthylamine were added. A colour change to red indicated a positive nitrate reduction and absence of a colour change would indicate there was no nitrate reduction or that further reduction of nitrite to ammonia or other compounds had occurred. In the absence of colour change, addition of small amount of zinc powder would catalyse nitrate to nitrite conversion and colour change to red, which would confirm a negative nitrate reduction.

2.2.6.1.10 *Voges-Proskauer (VP) Test*

The Voges-Proskauer (VP) test detects the production of non-acidic or neutral end product such as butanediol and acetoin (acetylmethyl carbinol) following glucose fermentation.

The VP broth was inoculated with fresh inoculum from a young culture and incubated at 50 °C for 24 h. To develop the test, α -naphthol (5 %) and potassium hydroxide (40 %) were added. A colour change to pink-burgundy indicated a positive test.

2.2.6.1.11 *Indole production*

The indole production test detects the ability of a bacterium to convert tryptophan into indole by degrading the amino acid tryptophan. To test for indole production, tube of tryptone was inoculated with a small amount of pure culture and incubated at 50 °C for 24 h. Then 5 drops of kovacs reagent [Conc. HCl (25 mL); iso-amyl alcohol (75 mL) and para-Dimethylaminobenzaldehyde (5 g)] was added directly to the tube. A positive indole test is indicated by the formation of a pink to red colour in the reagent layer on top of the medium within seconds of adding the reagent. The reagent layer remains yellow to lightly cloudy for a negative indole reaction. Most *Bacillus* species are indole negative.

2.2.6.1.12 *Arginine hydrolase test*

The arginine hydrolase test detects the ability of arginine hydrolase to release ammonium from arginine. The release of ammonium into the culture broth results in an alkaline medium. To perform the arginine hydrolase test, Peptone (0.1 g), NaCl (0.5 g), K₂HPO₄ (0.03 g), agar (0.3 g) were made up in 100 ml distilled water and autoclaved. This was allowed to cool to 55-50 °C and phenol red (0.001 g) and DL-Arginine HCl (1 g) were added and mixed. This was dispensed into test tubes and allowed to solidify. The tubes were stab-inoculated with bacteria from a fresh culture and tube covered with a few mL of sterile mineral oil, and incubated at 50 °C for 48 h. A deep pink colour indicated a positive reaction.

2.2.6.1.13 *Urea hydrolysis test*

The urea hydrolysis test detects the ability of urease to hydrolyse urea into ammonia (NH₄) and carbon dioxide (CO₂) i.e. $(\text{NH}_4)_2\text{CO} + \text{CO} \rightarrow \text{CO}_2 + 2\text{NH}_3$. Christensen's urea agar was prepared with peptone (1 g), dextrose (1 g), NaCl (5 g), potassium phosphate, monobasic (2 g), urea (20 g), phenol red (0.012 g), and agar (20 g). The urea base was prepared by mixing all constituents except agar in 100 mL distilled water and filter sterilised through 0.45 μm pore size filter. Agar was suspended in 900 ml distilled water, stirred to dissolve and autoclaved. Upon cooling to around 55 °C, the urea base was added and properly mixed. This was then dispensed into sterile tubes, slanted and allowed to solidify. The slant surface of the yellow-orange coloured medium was streaked with inoculums and incubated at 50 °C for 24 h. A colour change to bright pink indicated the hydrolysis of urea to ammonia.

2.2.6.1.14 *Effect of salt concentration and pH on N22 growth*

Salt concentration and pH are important physiological characteristics that affect cell growth and survival. To determine this effect, Nutrient agar plates with Sodium Chloride (NaCl) concentration (2-12 %) were prepared and inoculated with isolate N22 and observed for cell growth in 24 h. Also, nutrient broth (pH 4-13) were prepared and inoculated with single colony of N22 and incubated for 24 h. The cultures were plated on nutrient agar plates and observed for cell growth at 24 h.

2.2.6.2 *Molecular identification (phenotyping)*

Molecular phenotyping is the identification and classification of microorganisms according to their ribosomal DNA (rDNA) sequences. The distinct sequence information for individual microorganism enables identification up to species and strain level. 16S rDNA full gene sequencing was carried out with MicroSeqTM service (National Collections of Industrial Food and Marine Bacteria, UK). DNA was extracted from microbial culture and the 16S gene amplified by PCR. Cycle sequence analysis was performed on the ABI sequencer. The generated sequence was compared with sequence on the MicroSeqTM database and public databases to produce top sequence matches and a phylogenetic tree.

2.2.6.3 *Microbial spectral mass profiling*

Microbial spectral mass profiling is an emerging method for identifying microorganisms according to their unique characteristic mass spectral represented mainly by mass peaks of ribosomal proteins (Suh *et al*, 2005; Maier and Kostrzewa, 2007). Using Matrix-Assisted Laser Desorption and Ionisation Time-of-flight Mass Spectrometry (MALDI-TOF-MS) method, the protein molecules are co-crystallised with organic matrix (e.g. cyano-4-hydroxycinnamic acid), and the highly conjugated molecules are desorped into gas phase, undergoing little fragmentation, by pulsed laser (Guerrera and Kleiner, 2005; Kieman *et al*, 2007). The molecules are ionised (in the ionisation chamber) and separated according to their charge and mass (in the mass analyser). The emerging ion is detected as electrical signals by an electron detector, and the data processed and presented in height and intensity, according to their relative abundance, on a mass spectrum.

To generate microbial spectral mass profile, single colony of bacteria cultured on nutrient agar plate were extracted with 1 ml of acetonitrile and 70 % formic acid (1:1) mixture to obtain whole cell protein. The extracted protein sample (1µl) was conjugated with (1µl) α-cyano-4-hydroxycinnamic acid (HCCA) matrix (Sigma, USA) and analysed by MALDI-TOF MS (AXIMA CFR, Shimadzu Biotech) to generate the microbial spectral mass profile.

2.2.7 Optimisation of keratinase production

Several factors such as temperature, pH, and growth substrate significantly influence the synthesis of proteases in a fermentation culture (Singh *et al*, 1975; Wang and Shih, 1999; Yamamura *et al*, 2002). A proper balance of these factors results in optimum synthesis of specific proteases of interest. To determine the optimisation conditions for keratinase production, fermentation cultures were setup at temperatures (37 °C and 50 °C); pH (7, 8.5 and 10) and substrate concentration (0.8 %, 1.1 % and 1.4 %). Optimal pH was determined for culture pH 4 to 13 at incubation time of 10, 24 and 32 h. The final pH of the culture broths were measured at 32 h. All experiments were carried out in triplicates. Test of significant difference for the optimisation conditions in keratinase production was performed using One-way ANOVA. Spot inoculation assay was set up and incubated at 25 °C, 30 °C, 37 °C, 40 °C, 50 °C, 60 °C, 70 °C and 80 °C for 24 h to determine optimum temperature of growth and expression of keratinase activity.

2.2.8 Purification and characterisation of keratinase

2.2.8.1 Purification of keratinase

Crude culture supernatant normally comprises of complex mixture of enzymes, nucleic acid, cells and debris. Recovery of the biomolecules of interest is accomplished by separation and purification processes. Cells, debris and other solid particles are removed by centrifugation and filtration, and the cell-free culture concentrated and purified according to protein size, physico-chemical properties and binding affinity.

In order to purify keratinase, cell-free crude keratinase extract was concentrated in a Centriconplus-70 centrifugal filter device of molecular weight cut-off of 10 KDa (Millipore Inc.). The concentrated retentate was recovered after centrifugation at 4750 ×g for 45 min, and purified with the HiTrap Blue HP prepacked purification column (GE Healthcare Bio-sciences). The column material consists of resins (Blue sepharose) to which ligand (Cibacron TM Blue F3G-A dye) is covalently attached via the triazine part of the dye molecule, and has affinity for a wide range of enzymes. The column was equilibrated with 10 column volume of binding buffer (50 mM KH₂PO₄, pH 7.0). The concentrated supernatant sample was adjusted to the composition of binding buffer by diluting the sample with the binding buffer, and then applied to the column at a rate of 0.5 - 1 ml/min with a syringe and the fraction PSC was collected. The column was then eluted with the binding buffer (50 mM KH₂PO₄, pH 7.0) and the fraction BF collected and once the eluent became clear, the column was eluted with the elution buffer (50 mM KH₂PO₄, 1.5 M KCl, pH 7.0) and 5 ml fraction (EF) was collected.

A step-wise feedback purification model (Fig. 2.2) was developed to increase purification yield. The purification column was cleaned with 2 % alcohol after each purification cycle, washed through with the binding buffer and the fraction PSC (Fig. 2.2) re-introduced for subsequent purification cycle. Equivalent fractions of BF and EF from the three purification cycles were pooled, concentrated and characterised. The samples were then aliquoted and those for immediate use (up to 8 weeks) were stored at 4 °C and those for long storage at -38 °C.

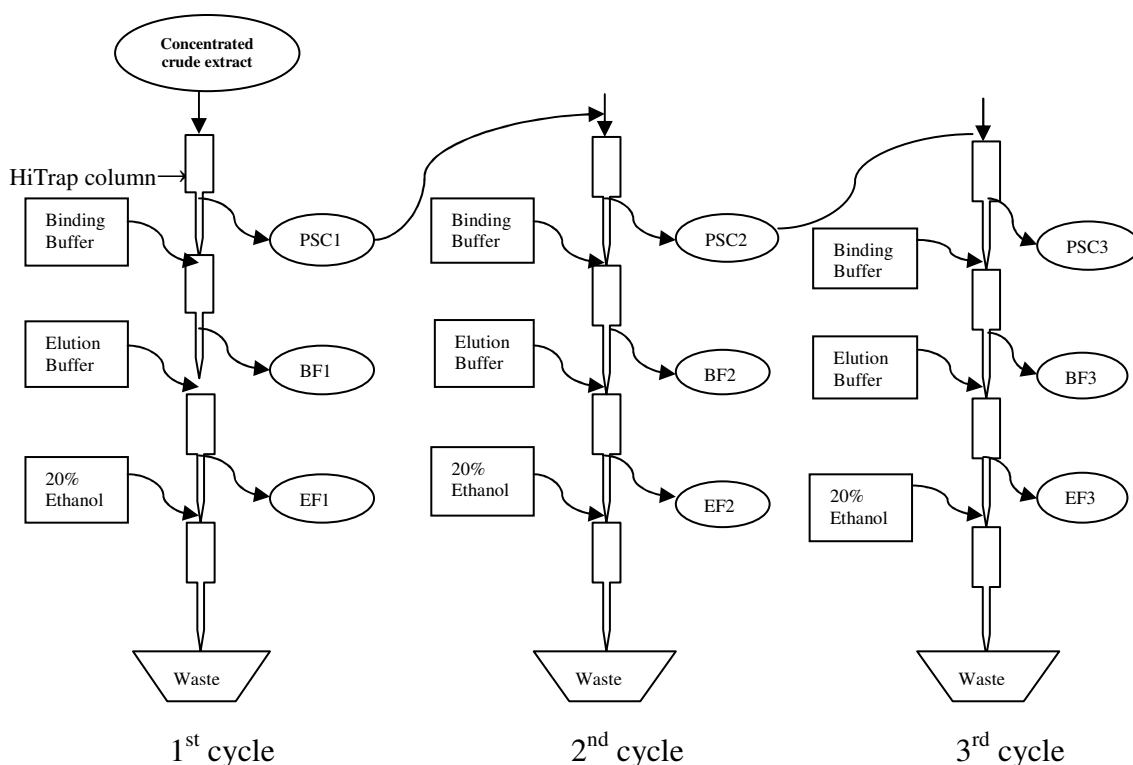


Fig. 2.2: Schematic diagram of the step-wise feedback purification model showing three purification cycles and the purification fractions PSC (collected after passing the concentrated crude supernatant through the column), BF (collected after flushing column with binding buffer) and EF (purified fraction collected after eluting column with elution buffer). The PSC fraction was re-introduced into the purification column during subsequent purification cycles. The numerical suffices indicate the number of purification cycle.

2.2.8.2 Characterisation of keratinase

2.2.8.2.1 Molecular weight

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) is a commonly used method for proteins separation and determination of molecular weight (Laemmli, 1970). The protein sample becomes negatively charged by attaching to SDS anions, so that the sample undergoing electrophoresis is separated according to the size (molecular weight) of the protein molecule, in which the smaller particles move faster through the gel.

To determine the molecular weight of purified keratinase, a 12 % gel was prepared as follows: The stacking gel (3 % polyacrylamide) solution contained H₂O (3.65 ml); stacking buffer (1.5 ml); protogel (0.8 ml); 10 % ammonium persulphate (APS) (37.5 µl); and Tetramethylethylenediamine (TEMED) (5 µl), and the resolving gel (12 % polyacrylamide) solution composed of H₂O (6 ml); resolving buffer (4 ml); protogel (6.25 ml); 10 % APS (75 µl); and TEMED (10 µl). The casting chamber was set up and the bottom and corners sealed with 1 % agarose. The resolving gel solution was added to cover $\frac{3}{4}$ of the cast and topped with distilled water. The gel was allowed to set for about 35 min and the water drained. The stacking gel was added to fill the cast to the top and well comb inserted and the gel allowed to set. The electrophoresis chamber was set up with the gel in place and the chamber filled with running buffer. The comb was removed to reveal the lanes and the protein sample (10 µl) gently loaded with fine long pipette placed close to the bottom of the wells. The electrophoresis unit was run at 150 V (constant voltage) and 200 mA for 50 min to separate the protein sample. The gel was carefully removed and placed in a plastic tub and the staining solution added and allowed to shake overnight at 35 rpm. The staining solution was decanted and destaining solution added and microwaved for 5 min. This was repeated once and destaining continued with shaking at 35 rpm until gel was sufficiently clear and the molecular weight bands clearly visible. The gel was then drained and sandwiched in transparent paper and scanned. Molecular weight band was traced against standard molecular weight markers (15 KDa-120 KDa).

To determine the molecular weight of the protein sample by MALDI-TOF MS, the purified keratinase sample (1 µl) was conjugated with 1 µl of sinapinic acid matrix (Sigma-Aldrich, USA) on a metal plate and allowed to crystallise at room temperature. Mass spectrum was acquired by MALDI-TOF MS (AXIMA CFR, Shimadzu Biotech) set on linear mode and power of 90 and with minimum smoothing.

2.2.8.2.2 *Protein concentration*

Protein concentration is a measure of protein abundance in sample solution, and is also used for assessing the success of a purification process and alongside enzyme activity can be used to calculate specific activity. Protein concentration is commonly measured by the Bradford Reagent method which is based on the binding of Commassie Brilliant Blue dye to protein resulting in a colour change that is quantified by spectrophotometry (Bradford, 1976). The dye donates free electron which interact with ionisable groups of the protein, causing disruption of the proteins primary structure and exposure of its hydrophobic pockets. These pockets bind to the non-polar regions of the dye, bringing the positive amine

groups close to the negative charge of the dye, and thus stabilises the blue dye. The amount of complex formed in the solution is measured as the protein concentration.

To measure protein concentration, protein sample (0.1 ml) was mixed with 3 ml of Bradford reagent and incubated at room temperature for 20 min at room temperature. The sample mixture was transferred to semi-micro cuvettes and the absorbance measured spectrophotometrically at 595 nm. The concentrations of the samples were derived using standard calibration curve prepared with Bovine Serum Albumin (BSA) of concentration range of 0-2 mg/ml.

2.2.8.2.3 *Peptide mass fingerprinting of keratinase*

Peptide mass fingerprinting is an analytical technique in which protein is cleaved into peptides which are measured according to their mass, and their specific peptide sequence used to identify and characterise protein. Purified keratinase was digested with 0.1mg/ml of trypsin (Sigma, UK) at 37 °C for 1 h. The digested sample (1 µl) was conjugated with 1 µl of sinapinic acid matrix and analysed by MALDI-TOF MS to map tryptic peptide mass fingerprints. The resulting mass spectrum was searched on mascot (Matrix Data Science) to identify possible matches.

2.2.8.2.4 *pH condition for optimum keratinase activity*

Optimum pH condition of keratinase is the pH at which its highest activity is achieved. To determine the optimum pH condition for keratinase, the reaction buffer for keratinase assay was adjusted to pH 7.5, 8.5, 9 and 10, and the activity of crude keratinase measured as described in section 2.2.5.1.

2.2.8.2.5 *Thermal stability of keratinase*

Enzymes can easily lose their activity under thermally unfavourable conditions. To determine the thermal stability of this keratinase, purified sample was stored at 4 °C for up to 8 weeks and the keratinase activity measured at intervals over the period to determine residual activity and thus the stability of the enzyme.

2.2.8.2.6 *Effects of chemical agents on keratinase activity*

Chemical agents such as Ethylenediaminetetraacetic Acid (EDTA), Dithiothreitol (DTT) and Sodium dodecyl sulphate (SDS) and Triton X-100 affect the activity of different keratinases variably. To determine their individual effect on crude keratinase, 1ml of EDTA (5 mM), DTT (5 mM), Triton X-100 (0.1 %) and SDS (0.1 %) were each mixed separately with 1 ml of crude keratinase and incubated at 50 °C for 15 min. The residual keratinase activity was then measured using 0.2 ml of the pre-incubated samples in keratinase assay (section 2.2.5.1). The sample that was used in the control experiment

(crude keratinase without any agent) was prepared the same way with crude keratinase and Tris HCl (pH 8.5) buffer (reaction buffer of the keratinase assay). Similar volumes were used to ensure equal dilution of the crude keratinase and they were also pre-incubated under similar conditions.

2.2.9 Production and characterisation of biosurfactant

2.2.9.1 Production of biosurfactant

Rhamnolipid biosurfactant of *Pseudomonas aeruginosa* NCIMB 8626 was produced in the Basal Mineral Medium (BSM) as described in Zhang *et al* (2005). The BSM was composed of (g/l): Sodium nitrate (NaNO_3 , 4.0); Sodium chloride (NaCl , 1.0); Potassium chloride (KCl , 1.0); Calcium chloride dehydrate ($\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ = 0.1); Dihydrogen potassium phosphate or phosphoric acid (KH_2PO_4 , 3.0); Di-sodium hydrogen orthophosphate 12-hydrate ($\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$, 3.0); Magnesium sulfate (MgSO_4 , 0.2); Iron (II) sulfate or ferrous sulphate ($\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.001), and trace element stock solution, 2 ml. The trace element stock solution was composed of (g/l): Iron (III) chloride hexahydrate or Ferric chloride ($\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$, 0.08); Zinc sulphate heptahydrate ($\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.75); Cobalt (II) chloride 6-water ($\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.08); Copper sulphate pentahydrate ($\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.075); Manganese sulfate monohydrate ($\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.75); Boric acid (H_3BO_3 , 0.15); Sodium Molybdate Dihydrate ($\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, 0.05); Sodium hydroxide (2 N NaOH) (adjusts BSM to an initial pH 7.0).

Pseudomonas aeruginosa (NCIMB 8626) was maintained in BH broth and incubated at 37 °C for 24 h at 150 rpm. The cells were washed twice in BSM by centrifuging the culture broth at 3000 rpm for 10 minutes, and then re-suspended in 40 ml of BSM. 0.6 ml of sterile glycerol was added and the preculture was incubated for 48 h. 250 ml of BSM in a 1 L Erlenmeyer flask was inoculated with 2 ml of the preculture. 6 ml of glycerol was added and the flask incubated at 37 °C, 150 rpm for 5 days. The culture broth was centrifuged at 4750 g for 20 min twice and the supernatant ultra-filtered under pressure using 0.45µm membrane filters (Millipore, UK) to obtain cells-free supernatant (crude biosurfactant extract).

2.2.9.2 Characterisation of biosurfactant

The protein concentration of the crude biosurfactant extract was measured by the Bradford reagent method as described in section 2.2.8.2.2. The biosurfactant concentration was measured by the orcinol method for determination of rhamnose (Chandrasekaran and Bemiller, 1980). Biosurfactant (200 µl) sample was mixed with 1.8 ml solution containing 0.19 % orcinol (orcinol monohydrate, Acros, USA) made up in 53 % H_2SO_4 . The mixture

was boiled in hot water bath for 20 min. After cooling at room temperature for 15 min, the samples were vortexed and the absorbance measured at 421 nm. Rhamnose standard curve was prepared with L-rhamnose (L (+)-rhamnose monohydrate from Acros) for a range 0-1 mg/ml to determine the rhamnose equivalent of the biosurfactant. The rhamnose values can be expressed as rhamnolipid values by multiplying them with a coefficient of 3.4 obtained from the correlation of pure rhamnolipid/rhamnose (Chandrasekaran and Bemiller, 1980).

2.2.9.2.1 Effect of biosurfactant on keratinase activity

To determine the effect of the crude biosurfactant on keratinase activity, biosurfactant was added to crude keratinase and the procedure performed as described in section 2.2.8.2.6.

2.3 Degradation of keratin substrates and scrapie prions by keratinase

2.3.1 Degradation of keratin azure

Characteristically, keratinases are able to degrade keratin azure by cleaving keratin causing release of azure dye into solution which is measured as absorbance change. To determine the ability of crude keratinase to degrade keratin azure, a test-tube containing 0.01g of treated keratin azure in 3 ml of 10 mM Tris HCl was inoculated with 1 ml of crude keratinase extract and incubated at 50 °C for 24 h. The reaction mixture was then physically observed for the release of azure dye into the solution. In the control experiment, the crude keratinase extract was denatured by boiling to destroy its activity.

2.3.2 Degradation of melanised feather

Melanised feather are highly resistant to degradation by keratinases than white feathers due to melanin pigment reported to inactivate keratinase (Gunderson *et al*, 2008). Therefore, keratinases that degrade melanised feather are potentially able to degrade highly stable and resistant keratins or keratin-like materials. To investigate the keratinolytic activity of N22 keratinase, native (non-autoclaved) melanised feathers (dove feather) were trimmed (4 cm x 2 cm) and washed twice with sterile distilled water, oven dried at 50 °C and weighed. The final wash effluent was inoculated on nutrient agar plates and incubated at 50 °C to check for possible microbial presence. The feathers were incubated at 50 °C overnight and 250 rpm in 8 ml of sterile distilled water and 2 ml of crude keratinase and compared to the negative control containing 10 ml of distilled water. A further experiment was carried out to compare the activity of denatured and normal crude keratinase. The enzyme was denatured by boiling for 30 min. The crude and denatured enzyme (10 ml) was each added to the melanised feather as described above. All experiments were carried out in duplicates. The extents of degradation of the feather substrates after 48 h were evaluated

by visual observation and quantitatively by measuring the residual dry weight by the weight loss method.

2.3.3 Degradation of scrapie prion by keratinase

2.3.3.1 Materials

Mouse-adapted ME7 scrapie infected brain homogenate (IBH) was used in this study for *in vitro* and *in vivo* experiments. The scrapie prion BH was obtained from Drs Stephen Whatley and Oduola Abiola at the Institute of Psychiatry (IOP), King's College London. Normal brain homogenate (NBH) from the batch of mice used for bioassay in this study was harvested and homogenised in phosphate buffered saline (PBS). All scrapie degradation experiments were conducted at Prof. Roger Morris Laboratory at the Wolfson Centre for Age Related Disease (CARD), King's College London, Guy's Campus. Handling of infectious scrapie material was carried out according to the World Health Organisations infectious control guideline for transmissible spongiform encephalopathies (WHO, 1999: <http://www.who.int/csr/resources/publications/bse/whocdscsraph2003.pdf>).

The following monoclonal primary antibodies were used to detect PrP^{Sc} in Western Blot analysis: SAF83 (SPI-Bio, Massy, France), and 98A3, 9A2, 12B2, and 6C2 (Courtesy of Dr Jan Langeveld, Central Veterinary Institute of Wageningen UR, The Netherlands). SAF83 (IgG1):[from hamster brain SAF (scrapie associated fibrils)] in mouse host, recognises solid-phase immobilised peptide 126-164. Monoclonal antibody 98A3 (IgG2a) was elicited with recombinant bovine PrP and recombinant human PrP mapped to KRPRKP. The antibody 9A2 (IgG1) was elicited with bovine peptide of N-terminus PrPres boPrP97-115 (GGGGWGQGGTHGQWNKPSK) mapped to WNK. 12B2 (IgG1) was elicited with bovine peptide of N-terminus PrPres boPrP97-115 (GGGGWGQGGTHGQWNKPSK) mapped to WGQGG and 6C2 (IgG2b) was elicited with bovine peptide boPrP 117-130 (KTNMKHVAGAAAAG) mapped to HVAGAAA. All mappings described above were performed with pepscan (Dr Jan Langeveld, Personal communication).

2.3.3.2 Western blot

Western blot (WB) is a commonly used biochemical method for the immunodetection of proteins using specific antibodies after the protein sample have been separated by gel electrophoresis and transferred onto membrane (Kurien and Scofield, 2006). WB analysis was used to establish the presence or absence of prion protein in brain homogenate samples, and hence to evaluate the enzymatic degradation of scrapie prion.

2.3.3.2.1 *Sample preparation and gel electrophoresis*

Brain homogenate (5µl) containing either 1 % normal brain homogenate (NBH) or 1 % ME7 scrapie infected brain homogenate (IBH) were mixed with 5µl PBS, or 10 µl brain homogenate (specified as used), and incubated with proteinase K (PK), keratinase or keratinase combined with biosurfactant at digestion times (10 min to 2 h) as specified for each experiment. The digestion was terminated by addition of 1 µl of 50 mM Phenyl methyl sulfonyl fluoride (PMSF) followed by the addition of 11 µl of 2× sample buffer and heated at 100 °C for 10 min on a dry block (Techne). Digested samples (10 µl) were run on SDS-PAGE gel as described in section 2.2.8.2.1.

2.3.3.2.2 *Transfer of sample to membrane*

Polyvinylidene fluoride (PVDF) membrane was briefly soaked in methanol and then placed on filter paper which was immersed in transfer buffer (composed of glycine, 2.9 g; Tris, 5.8 g; SDS, 0.7 g; and methanol, 200 ml, made up to 1000 ml with distilled water). The separated protein bound-SDS PAGE gel was placed on the PVDF membrane and carefully sandwiched between filter papers. The sandwich was wrapped with tissue paper and gently pressed together with a roller to drain excess transfer buffer and to remove trapped air bubbles. The sandwich was placed in a transfer cell [Trans-Blot SD, semi-dry transfer cell (Biorad)] and run at 15 V and 200 mA for 50 min for a single membrane transfer.

2.3.3.2.3 *Immunodetection*

After the transfer, the membrane was carefully removed and blocked with 5 % skimmed milk (incubated for 1 h at 30 rpm), and then probed with the primary antibody [SAF 83 (0.2 µg/ml)] in 1 % skimmed milk (1:5000) and incubated for 1 h at 30 rpm. Primary antibodies such as 98A3 (5 µg/ml), 9A2 (0.5 µg/ml), 12B2 (0.2 µg/ml) and 6C2 (1 µg/ml) were also used in order to determine the antibodies of most affinity to the linear epitopes of the ME7 scrapie prion. The membrane was quickly washed twice with 0.1% Tween in 1× PBS (PBST) followed by six 5-min washes. The membrane was then probed with secondary antibody (Amersham ECL Anti-mouse IgG Horseradish peroxidase linked whole antibody (from sheep); GE Healthcare, UK) in 1 % skimmed milk (1:5000) and incubated at room temperature, 30 rpm for 50 min and washed as described above. 5 % Bovine Serum Albumin (BSA) was also used as membrane blocking buffer (incubated for 1 h at 30 rpm) in order to determine the most suitable blocking agent that will not cross react with keratinase and will block undesirable background signals.

Prion protein was detected by using Amersham™ ECL™ Plus Western Blot Detection System (GE Healthcare, UK). Lumigen PS-3 detection solutions: A (1.5 ml) and B (37.5

μl) were mixed and poured over the membrane which had been drained and placed on foil wrapped glass plate, and allowed to stand for 5 min. The secondary antibody conjugate, Horseradish peroxidase (HRP) catalyzed the oxidation of luminol in the presence of hydrogen peroxide, and the luminol decays via a light-emitting pathway, and was photographed on Kodak film, and developed with Mediphot 937 X-ray film processor (Colenta Labortechnik, Austria).

2.3.3.3 *Optimisation of enzymatic degradation of scrapie prion*

Optimisation of an enzymatic degradation procedure enables the identification of suitable degradation conditions. To optimise the enzymatic degradation of ME7 scrapie, factors such as the state and concentration of keratinase, digestion temperature, incubation time, and the addition of detergents were investigated as reported below.

2.3.3.3.1 *Degradation with keratinase fractions and enzymatic composition*

ME7 scrapie brain homogenate (5 μl) samples mixed with 5 μl PBS were digested separately with 1 μl of crude keratinase extract (S), concentrated crude keratinase (CS), and PSC, BF and EF collected as fractions from the purification procedure and incubated at 50 °C for 2 h to determine the most effective fractions for degrading ME7 scrapie prion. A replicate experiment was conducted with the keratinase fractions mixed with 1 μl of crude biosurfactant extract (BS) (EF+BS) and digested at 50 °C for 1 h to determine the effect of biosurfactant on ME7 prion degradation. Furthermore, ME7 scrapie brain homogenate (10 μl) was digested at 50 °C for 2 h with the enzymatic composition (EF+BS). Digestions of ME7 by BS, EF and EF+BS at 65 °C for 1 h were carried out. All control experiments were not enzyme digested.

PrP^C is immunologically indistinguishable from PrP^{Sc}, but it is degraded by proteinase K (PK). In the degradation of normal brain homogenate or degradation of infectious brain homogenate to digest PrP^C (control experiments), PK was used at the final concentration of 10, 50, 77 or 100 μg/ml.

2.3.3.3.2 *Optimisation of enzymatic composition and incubation conditions*

ME7 scrapie sample was digested with EF+BS in which EF composite was diluted in PBS as 1:50; 1:100; and 1:200. Also, to optimise the incubation time, ME7 scrapie BH (10μl) was digested at 50 °C for 30, 45, 60, 90 and 120 min with EF (1 μl) +BS (2 μl). Digestions were also carried out at 65 °C for 10, 30 and 45 min.

2.3.3.3.3 *Degradation of other scrapie prion samples*

In order to investigate the efficacy of EF+BS on other scrapie strains, brain homogenate samples (1%) of ME7, PG 300/97, SSBP/1, PG 2413/98, PG 467/07, PG 241/97 scrapie

prions (Kindly provided by Animal Health and Veterinary Laboratories Agency, UK) were digested with EF+BS at 65 °C for 1 h, and analysed by Western blot for PrP^{Sc}.

2.4 Residual infectivity evaluation of keratinase-digested scrapie prions

2.4.1 Standard scrapie cell assay (ex vivo assay)

The standard scrapie cell assay (SSCA) is an important quantitative, highly sensitive cell-based infectivity assay for characterising scrapie prions using suitable susceptible cells (Klohn *et al*, 2003). Prion infection of cells have been shown to occur within 1 min of exposure (Goold *et al*, 2011). Susceptible cells infected with scrapie prion accumulate PrP^{Sc} and infectivity, hence SSCA was used to evaluate residual infectivity of enzyme-digested SSBP/1 scrapie prion infected brain homogenate. The SSCA was carried out at Animal Health and Veterinary Laboratories Agency, New Haw, Weybridge, UK with Michael Neales who have a lot of experience with SSCA and performed as described in Neale *et al* (2010).

2.4.1.1 Cell line and culture medium

Sheep scrapie brain pool/1 (SSBP/1) scrapie susceptible Rov9 cells (Kindly provided by Animal Health and Veterinary Laboratories Agency, UK) were used in the SSCA as ME7 scrapie susceptible cells were unavailable. The Rov9 cells were maintained in Eagle's minimal essential medium (EMEM, Gibco) supplemented with 10 % fetal calf serum and 2 % HEPES. Rov9 infection was cultured in OptiMEM medium (Invitrogen) supplemented with 10 % serum (Gibco) and antibiotics/antimycotic (Penicillin, 100 units; Streptomycin, 100 µg and Amphotericin B, 0.25 µg).

2.4.1.2 Infection of cells with SSBP/1

Approximately 20000 susceptible cells in 180 µl medium/well were plated wells of a 96-well plate and cultured for 16 h (to allow cells to adhere) at 37 °C in a humidified 5 % CO₂ atmosphere in a cell culture incubator. 20 µl of 1:10 dilution of inocula was further diluted to 10⁻⁴ concentration and added to the cell culture. The inocula were 1 % SSPB/1 digested with EF, BS and EF+BS at 65 °C for 1 h and the reaction was terminated with PMSF. The positive controls included incubated and non-incubated SSBP/1 to monitor the effect of incubation temperature. The cells were cultured for 3 days, trypsinised with 60 µl ATV [standard trypsin (0.05 %)/EDTA (0.02 %)] at 37 °C to detach cells, and split 1:6 into fresh 96-well plates. The cells were split every 3 days for three times and grown to confluence following the third split.

2.4.1.3 *ELISPOT Assay*

Enzyme-linked immunospot assay (ELISPOT) plate (Multiscreen-IP filter plates, Millipore) was activated with 50 µl of ethanol (70 %) added to each well and suctioned off. Each well was washed twice with 160 µl PBS. 100 µl of media containing 10 % serum was added to each well to inhibit trypsin in the cell suspension and to prevent wells from drying out while trypsinising (at 37 °C) to detach cells. Cells in the 96-well plate were trypsinised with 60 µl ATV in each well at 37 °C and ensuring that cells were detached by tapping the plate and pipetting vigorously but avoiding foam. 10 µl of the cells was passaged into fresh media in 96-well plates to maintain the challenge and 30 µl of the cells were plated into the ELISPOT plate. PBS (180 µl) was added to the remaining cells and 30 µl plated into Trypan blue plate. The plates were drained by vacuum and the wells allowed dry for 1 h. The ELISPOT plate was briefly digested with 60 µl of PK (10 µg/ml) solution prepared in lysis buffer (50 mM Tris HCl (pH8), 150 mM NaCl, 0.5 % Na deoxycholate, 0.5 % Triton X-100, H₂O) and incubated at 37 °C for 30 min. The PK was vacuumed off and the wells washed twice with 160 µl PBS on vacuum. 160 µl of 1 mM PMSF was added to each well to stop the reaction and vacuumed. 120 µl Tris-guanidinium thiocyanate (GSCN) was added and plate incubated at room temperature for 10 min and the GSCN flickered off onto absorbent paper in an autoclave bag and the wells washed with 160 µl PBS (4 washes) and another 3 washes on vacuum.

2.4.1.4 *Immunodetection*

Blocking buffer (120 µl of superblock per well) was added to the ELISPOT plate and the plate incubated at room temperature for 1 h and suctioned off. 60 µl of anti-PrP (Sha31) in TBST/ 1 % milk powder was added and incubated for 1 h at room temperature and the supernatant discarded. The wells were washed 4 times with 160 µl TBST and flickered off. 60 µl goat anti-mouse alkaline phosphatase conjugated in TBST/ 1 % milk powder was added to each well and incubated for 1 h at room temperature, and the supernatant discarded. The wells were washed 4 times with 160 µl TBST and flickered off. The plastic underdrain was removed and the membrane dried by pressing the bottom of the wells against a tissue, and the plate dried under a hood at room temperature. Alkaline phosphatase dye (Alkaline phosphatase conjugate substrate kit, Sigma) prepared according to manufacturers instruction was added to each well (54 µl) of the filter plate and incubated for 16 min at room temperature while avoiding air bubbles on the filter membrane. The supernatant was discarded and the wells washed twice with 160 µl water and the supernatant discarded. The plate was completely dried and the infected cells were visualised and read on plate reader. Positive cells (spots) were counted using a Zeiss KS-ELISPOT imaging system running Wellscan software (Image Associates); the images were

photographed.

2.4.1.5 Determination of cell number with trypan blue assay

The trypan plate prepared and dried in 2.4.1.3 containing 1:10 of the cells used in the ELISPOT assay was stained by placing the plate on vacuum device and flushing the wells with 100 µl 0.04 % Trypan Blue in lysis buffer (a few seconds exposure). The wells were rinsed twice with 160 µl PBS and dried. Cells were counted using the ELISPOT equipment.

2.4.1.6 Analysis of scrapie cell culture assay

Bar chart of the number of spots detected (infected cells) were plotted for the treatment groups: IBH, Incubated IBH, EF, BS and EF+BS. Test of difference in the number of infected cells in the EF, BS, EF+BS and IBH groups were compared with those of the incubated IBH group using Two-Sample T-Test.

2.4.2 Mouse bioassay (in vivo assay)

Mouse bioassay is an experimental method for transmission of infectivity to recipient animals and is considered as the gold standard for residual prion infectivity evaluation and validation of prion decontamination methods (Collinge, 2001). A normal (healthy) mouse challenged with the prion agent is expected to become infected and eventually die of the disease. The absence of clinical signs of disease and PrP^{Sc} in hosts challenged with degraded prion inoculum would imply complete degradation of the infectious agent. To test for residual infectivity and transmission of disease, mice were inoculated with enzyme-digested ME7 scrapie inoculum and observed for clinical signs of disease, and analysed for PrP^{Sc}.

2.4.2.1 Preparation of mice and experiment setup

Mouse bioassay for evaluation of residual infectivity was conducted at the animal facility at Biological Services Unit, Franklin Wilkins Building, King's College London. All animal experiments were performed in compliance with standard procedure for use of animals, and according to UK Home Office Animal (Scientific procedure) Act 1986. Transgenic (Tga20) mice (courtesy of Prof. Charles Weissmann) overexpressing PrP^C were outbred for 6 generation to C57BL/6 mice. The mice used for the bioassay were selected by immunohistochemistry method. Freshly clipped ears and tails were sectioned by cryostat onto poly-lysine coated slides and allowed to air-dry overnight. The slides were rinsed with 5 % milk PBS buffer and 2S Alexa-Fluor-594-Fab applied for 15 min and then washed 3 times with 1 % milk PBS. The samples were fixed with 4 % paraformaldehyde (PFA) and viewed under fluorescent microscope to detect PrP^C (positive staining). Positive and

negative control tails were included in the experiments. Selection of animals was carried out by Angela Jen at Prof. Roger Morris' Laboratory, Kings College, London.

The experimental setup of the mouse bioassay (Table 2.1) shows the different treatment groups according to mice groups injected with normal brain homogenate (NBH), ME7 infected brain homogenate (IBH), log-dilutions of IBH or IBH digested with the enzyme preparation (EF+BS) at 65 °C for 1 h. Brain homogenates were used as 1 % and all mice were inoculated intraperitoneally with 100 µl inocula except for the IBH (1:10 dilution) group which were inoculated with 70 µl in order to inoculate more mice. All inoculations were carried out by Prof. Roger Morris in compliance with the UK Home Office Animal (Scientific procedure) Act 1986.

Table 2.1: Experimental layout of mice bioassay including the treatment groups and the number of mice inoculated.

Treatment groups		No. of mice inoculated
Inoculated with normal brain homogenate (NBH)	Negative control	5
Inoculated with infectious brain homogenate (IBH) Neat	Positive controls	8
Inoculated with infectious brain homogenate (IBH) 1:10 dilution		9
Inoculated with infectious brain homogenate (IBH) 1:100 dilution		13
Inoculated with infectious brain homogenate (IBH) 1:1000 dilution		6
Inoculated with infectious brain homogenate (IBH) digested with enzymatic treatment (EF+BS)	Experimental group	15

2.4.2.2 Analysis of bioassay

Prion infected mice characteristically accumulate PrP^{Sc} and prion infectivity in their brain, hence, detection of PrP^{Sc} is an important parameter to determine if challenged mice have been infected. Survival times post inoculation were recorded when challenged mice died or developed signs of clinical disease and were culled. Their brains were harvested and homogenised in sterile phosphate-buffered saline (PBS) as 1 % (w/v) brain homogenates. Samples were digested with PK (77 µg/ml final concentration) and analysed for presence of PrP^{Sc} in the brains by western blot. Only mice that were dead or culled at ≤ 550 days

and those that showed positive PrP^{Sc} were included in the survival time analysis. Boxplot of the individual and mean survival times for the treatment groups were plotted. Test of difference in survival times for the different treatment groups were analysed by One-Way ANOVA. Test of significant increase in the survival times for the IBH-digested group compared to IBH neat group was analysed by Two-Sample T-Test.

2.4.2.3 *Comparing PrP^C expression in C57BL/6 mice and Tga20 mice used for bioassay*

The Transgenic (Tga20) mice used for the mice bioassay showed survival time characteristic of C57BL/6 (wild-type) mice and hence were compared for their levels of PrP^C expression. Tga20 overexpressing PrP^C propagates prion disease faster than wild type mice as a result abundant PrP^C substrate resulting in shortened incubation time of disease. The level of PrP^C in C57BL/6 (Charles River) brain homogenate sample was compared with 4 random samples of Tga20 mice used in this bioassay. Undigested brain homogenates (1 %) were analysed by western blot as already described. Two 1/3 dilutions of each sample was included in the gel. β -actin (Cell Signaling Technology) was used as internal control to check for uniformed loading of sample.

Chapter 3: Results

3.1 Isolation and characterisation of proteolytic microorganisms, keratinase and biosurfactant

3.1.1 Isolation of microorganisms from environmental samples

A total of thirty-two bacteria were isolated on feather meal agar from primary effluent, poultry and animal wastes. These isolates were selected and identified based on their colony morphology, size, shape, texture, structure, pattern and colour. Single colonies of the isolates were obtained by re-streaking on feather meal agar plates. The purified isolates stored in liquid nitrogen reached a cell density of up to 10^8 CFU/ml in 24 h when cultured in nutrient broth and plated on nutrient agar plates.

3.1.2 Screening of proteolytic microorganisms

3.1.2.1 Spot inoculation assay

The spot inoculation assay detects the proteolytic properties of bacterial strains by their ability to grow on casein-agar, hydrolyse casein and form hydrolysis ring. Results of screening test of isolate N22, compared to bacteria species; *Pseudomonas aeruginosa* NCTC 10662, *Escherichia coli* NCTC 10418, *Staphylococcus aureus* NCTC 6571, *Bacillus subtilis* NCTC 10400 and *Bacillus stearothermophilus* NCTC 10007 (Fig. 3.1) show that isolate N22 was highly proteolytic.

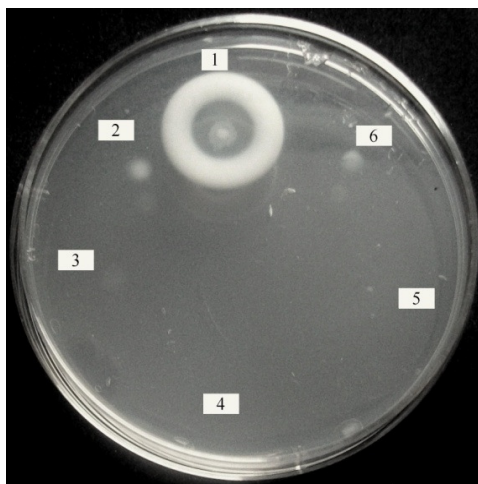


Fig. 3.1: Screening for proteolytic microorganisms by spot inoculation assay. Isolated strain N22 (1) shows higher proteolytic activity than *Pseudomonas aeruginosa* NCTC 10662 (2), *Escherichia coli* NCTC 10418 (3), *Staphylococcus aureus* NCTC 6571 (4), *Bacillus subtilis* NCTC 10400 (5) and *Bacillus stearothermophilus* NCTC 10007 (6).

3.1.2.2 *Hydrolysis ring assay*

Hydrolysis ring assay enabled screening of crude enzyme extract for proteolytic activity by their ability to hydrolyse casein. A validation experiment of hydrolysis ring assays showed an inverse correlation between casein ring formation and concentration of keratinase for crude keratinase dilutions of 1:0 (neat); 1:10; 1:100; 1:1000 (Pearsons correlation = 0.761, $p = 0.239$). The diameters of the hydrolysis ring were 20 mm, 16.3 mm, 12.5 mm and 6.3 mm respectively (Fig. 3.2).

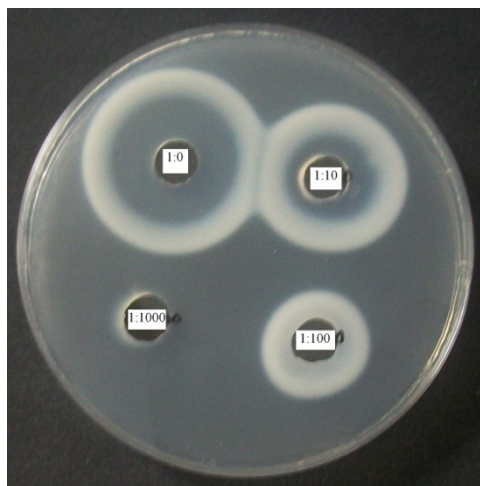


Fig. 3.2: Relationship between keratinase activity and hydrolysis ring. Clockwise from top left are inoculations with keratinase dilution of 1:0, 1:10, 1:100 and 1:1000 incubated at 50 °C for 24 h.

3.1.3 *Standardization of keratin azure substrate*

Standardisation of keratin azure (KA) substrate yielded a homogeneous substrate by removing excess azure dye and debris, thus enabled consistent and reliable keratinase activity measurement. In the treatment of KA, the highest dye release occurred when it was incubated with sterile distilled water as measured by the absorbance reading of 0.069 ± 0.030 compared to 0.022 ± 0.026 when incubated in 10 mM Tris HCl buffer. Distilled water was more efficient in removing loosely bound azure dye. However, in both cases, a large variation in absorbance changes occurred among sample replicates. In typical keratinase assay controls using unwashed and washed keratin azure, the mean absorbance \pm SD measured were 0.116 ± 0.020 and 0.046 ± 0.000 respectively (Table 3.1). The absorbance reading with the washed samples was not different from distilled water blank in the same batch of cuvette used. The large standard deviation for the unwashed keratin azure revealed significant disparity for the sample replicates compared to treated keratin azure. There was a significant difference in absorbance reading between treated (homogeneous) and untreated (heterogeneous) keratin azure ($p = 0.005$ at 95.5 % confidence interval; Mann-Whitney Test).

Table 3.1: Validation of standardisation protocol for the preparation of keratin azure (KA) substrate
Treated keratin azure is highly homogeneous with insignificant variation within sample replicates (n = 6), and is suitable as substrate for keratinase assay.

Substrate	Absorbance range	Mean absorbance \pm SD
Untreated KA	0.048	0.116 ± 0.020
Treated KA	0.002	0.046 ± 0.000

3.1.4 Screening for keratinolysis and selection of isolates

Keratinolytic isolates were specifically screened and selected by measuring the activity of their keratinases, and those that are potentially prion degrading were identified by their activity relative to proteinase K (PK) used to characterise prion. Results of keratinase activities of 32 isolates and a reference strain *Bacillus licheniformis* PWD-1 as evaluated by keratinase assay showed that isolate number 22 (N22) demonstrated the highest mean keratinase activity (Fig.3.3), and hence was selected for further study. Isolate N22 showed 58 % higher keratinase activity than the reference strain *B. licheniformis* PWD-1.

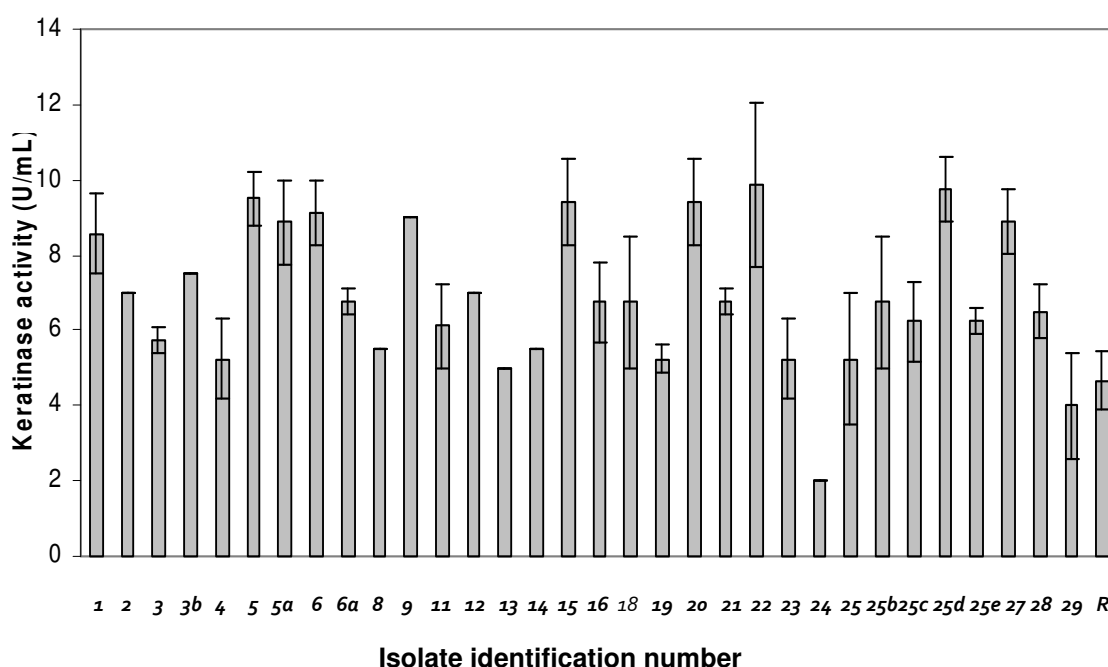


Fig. 3.3: Keratinase activity (Mean \pm SD, n = 3, each data point) for 32 microbial isolates tested by the keratinase assay using keratin azure as substrate. A number of isolates including number 22 showed significant activity relative to reference strain *Bacillus licheniformis* PWD-1 (Labelled R).

Keratinase activity equivalent to 0.2 mg/ml concentration of proteinase K determined from PK calibration curve (Fig. 3.4) was ≈ 5.9 U/ml, therefore, keratinase activity for crude culture extract of the isolates ≥ 5.9 U/ml were considered to be significant. PK of 0.2 mg/ml was reported to fully degrade prions under denaturing conditions to undetectable limit as assayed by Western blotting (Langeveld *et al*, 2003). Crude keratinase extract of isolate 22 showed 54 % more activity than 0.2 mg/ml of PK on keratin azure.

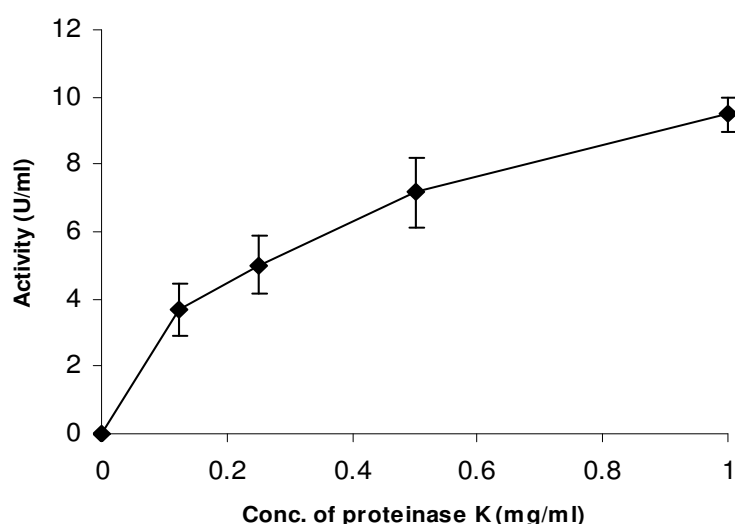


Fig. 3.4: Calibration curve for keratinase activity equivalent of Proteinase K degradation of keratin azure. PK concentration of 0.2 mg/ml is equivalent to 5.9 U/ml keratinase activity and was considered as a significant cut-off for keratinolytic microorganisms. Activity represents mean \pm SD (n = 5, each data point).

3.1.5 Identification and characterisation of strain N22

A number of morphological, physiological, biochemical and molecular properties were used to identify, classify and characterise bacteria isolates. Isolate N22 was found to be a Gram positive rod bacterium displaying a number of characteristics matching the *Bacillus* species listed in the Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1993; Table 3.2). This isolate was confirmed by full gene sequence analysis of the 16S rDNA with 99.93 % similarity to *Bacillus licheniformis* ATCC14580. Phylogenetic tree show isolate N22 within the same branch of *Bacillus licheniformis* species within the cluster of *Bacillus* species (Fig. 3.5). This isolate was named *Bacillus licheniformis* N22 strain and deposited with the National Collection of Industrial Food and Marine Bacteria (NCIMB) with designated ascension number NCIMB 41708. Mass spectral fingerprint for *Bacillus licheniformis* N22 and *Bacillus licheniformis* PWD-1 acquired by MALDI TOF-MS (Fig. 3.6) show highly stable and reproducible characteristic peaks.

Table 3.2: Morphological, physiological and biochemical characterisation of isolate N22

Tests	Response
Colony morphology	Rod-shaped, opaque, rough edge, mucoid, and domed surface, creamy-white colour,
Gram stain	+
Endospores formation	+
Anaerobic growth	-
Motility	+
Oxidase reaction	+
Catalase reaction	+
Triple Sugar Iron (TSI)	K/A*
Glucose	+
Lactose	-
Iron	-
Reduction of nitrates to nitrites	+
Voges-Proskauer (VP)	+
Indole production	-
Arginine DiHydrolase	+
Urease	+
Hydrolysis of casein	+
Onset of growth on nutrient agar plate (37 °C)	9 h
(50 °C)	5 h
Growth in nutrient agar at NaCl conc. 2-12 %	+
Growth in nutrient broth at pH 6-12	+

* K- alkaline reaction; A-acid production.

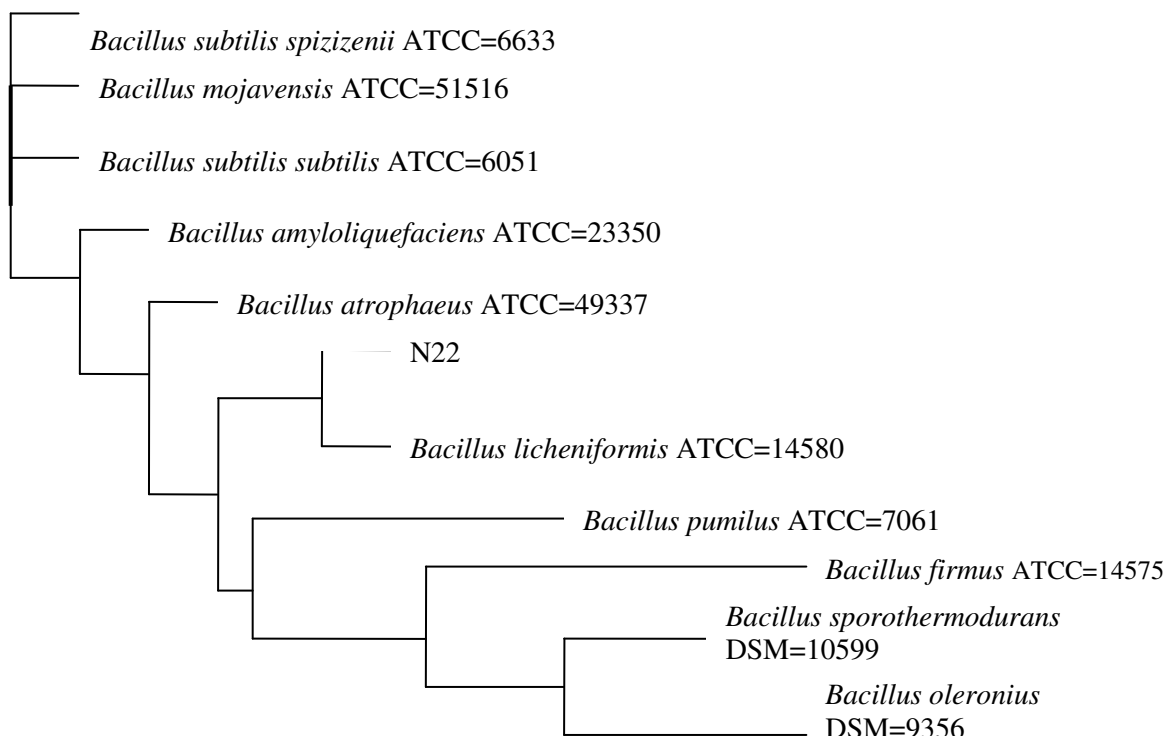


Fig. 3.5: Phylogenetic tree showing the top 10 hits generated from the 16S rDNA full sequence analysis of *Bacillus licheniformis* N22 within a cluster of *Bacillus licheniformis* specie.

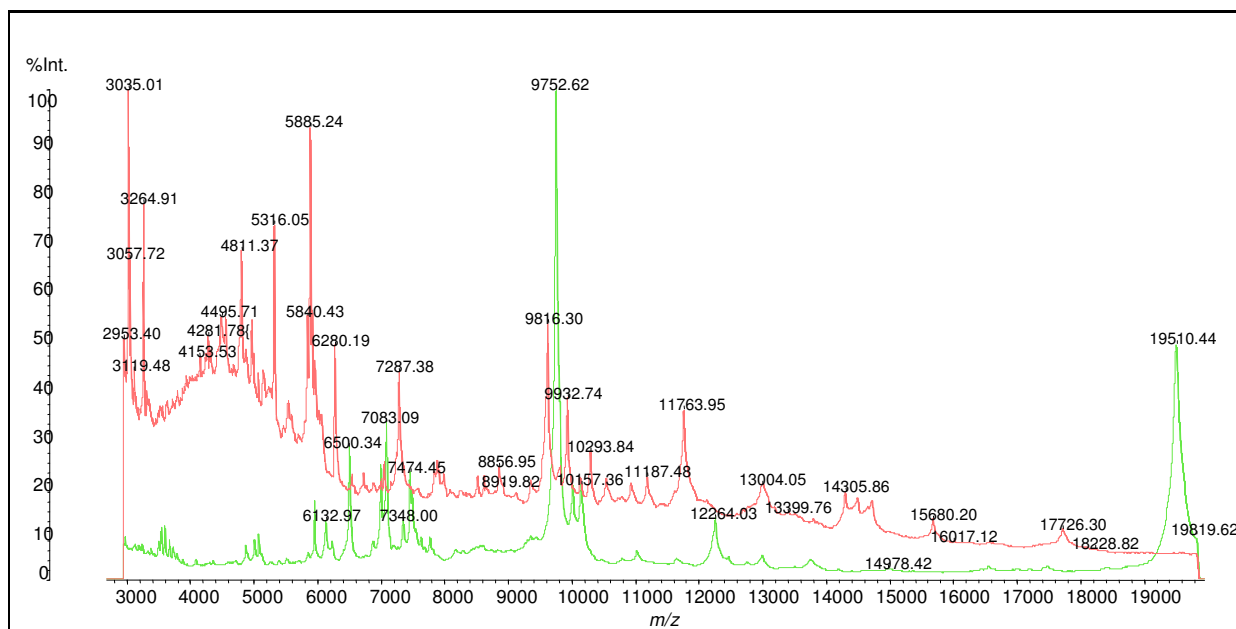


Fig. 3.6: Spectral mass fingerprint for *Bacillus licheniformis* N22 (red) and *Bacillus licheniformis* PWD-1 (green) showing distinct protein peaks, which are used for identifying bacterial strains.

3.1.6 Optimisation of keratinase production

Optimisation of physiological conditions of fermentation cultures such as temperature, pH and substrate concentration resulted in optimum keratinase synthesis and keratinase activity. The strongest casein hydrolysis ring expression by *Bacillus licheniformis* N22 in the spot inoculation assay was observed at 50 °C (Fig. 3.7), which is the optimum temperature for growth and production of keratinase. The relative hydrolysis ring diameter (%) at 25, 30, 37, 40, 50, 60, 70 and 80 °C were 0, 0, 33, 44, 100, 78, 67 and 44 % respectively, where the diameter at 50 °C was considered as 100 %.

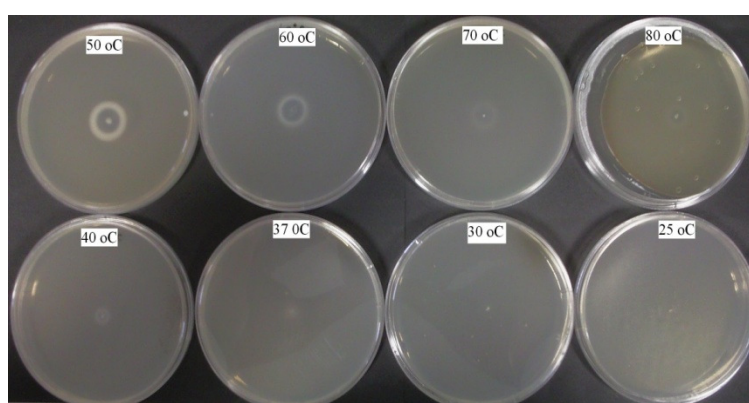


Fig. 3.7: Optimum temperature determination by casein-agar plate spot inoculation assay for temperature range 25 °C-80 °C. Casein hydrolysis was strongest at 50 °C which corresponds to its optimum temperature condition.

Keratinase production by *Bacillus licheniformis* N22 occurred over a wide pH range of 6-12, with a sustained optimum at initial culture pH 8-9 (Fig. 3.8) after incubation for 32 h.

One-way ANOVA for keratinase production in fermentation cultures at 10 h, 24 h and 32 h suggested that they were different ($p = 0.000$).

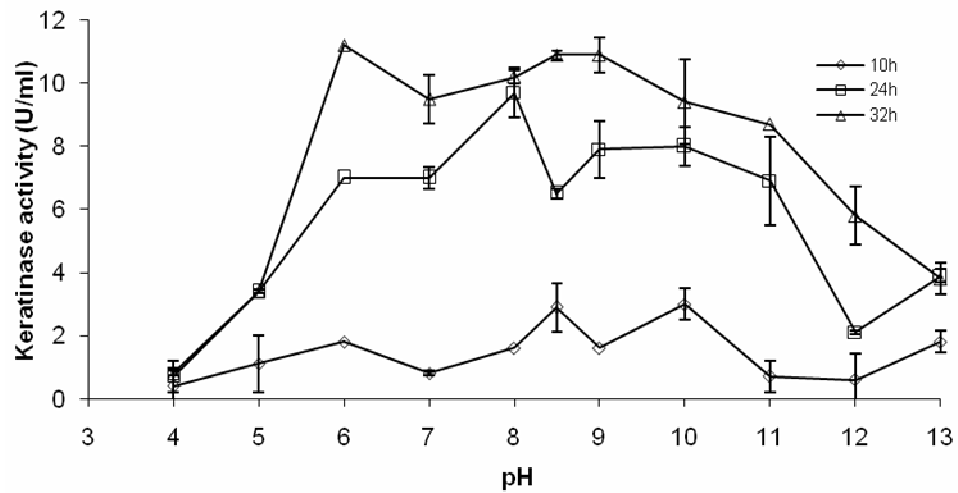


Fig. 3.8: Kinetics of keratinase activity (U/ml \pm SD; $n = 3$, each data point) showing the robustness of keratinase synthesis over a wide pH range (pH 6-10) at 32 h incubation time. Significant differences ($p = 0.000$; one-way ANOVA) existed for keratinase production at 10 h, 24 h and 32 h over a range of pH conditions.

The final pH of the fermentation culture broth was approximately pH 8.3 irrespective of the initial pH for pH (6-12) after incubation for 32 h (Fig.3.9). The pH 6-8 and pH 9-13 were up-regulated and down-regulated respectively, with those of pH 7-12 stabilising at final culture pH \approx 8.3, whereas initial culture pH ≤ 5 and ≥ 13 remained about the same at the end of 32 h incubation period.

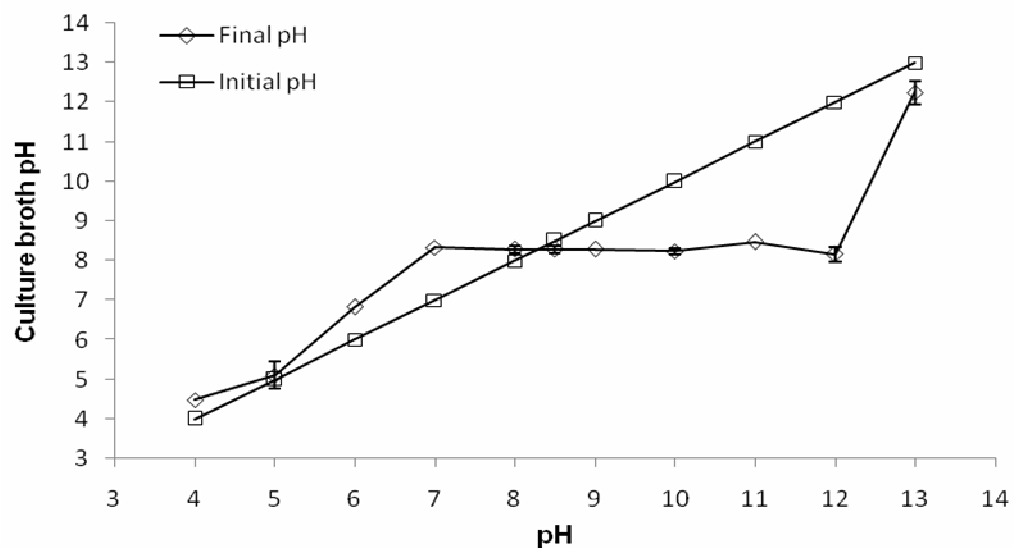


Fig. 3.9: pH kinetics in fermentation culture with final pH tending towards 8.3, which indicates the optimum pH for cell growth and synthesis of extracellular keratinase. The buffering effect was a response to metabolic products resulting from two possible pathways. Graph for final pH represents mean \pm SD ($n = 3$, each data point).

The optimal substrate concentration for production of keratinase of highest activity was found to be 1.1 % (w/v) resulting in keratinase activity of approximately 11 ± 0.71 U/ml. Higher substrate concentrations more generally tended to limit keratinase synthesis (Fig. 3.10). Generally, keratinase production was higher at 50 °C than at 37 °C, and also at pH 8.5 than at pH 7 or pH 10. Acidic pH ($\text{pH} \leq 4$) and temperature up to 63 °C were found to be unfavourable for cell survival and growth of this strain and hence for keratinase production. Keratinase synthesis was induced by feather meal substrate. Keratinase activity in bacterial culture grown in nutrient broth was significantly lower (3.5 U/ml) than that grown in minimum medium supplemented with feather meal substrate (11 U/ml). An extended fermentation culture incubation up to 52 h resulted in a 30 % reduction in keratinase activity.

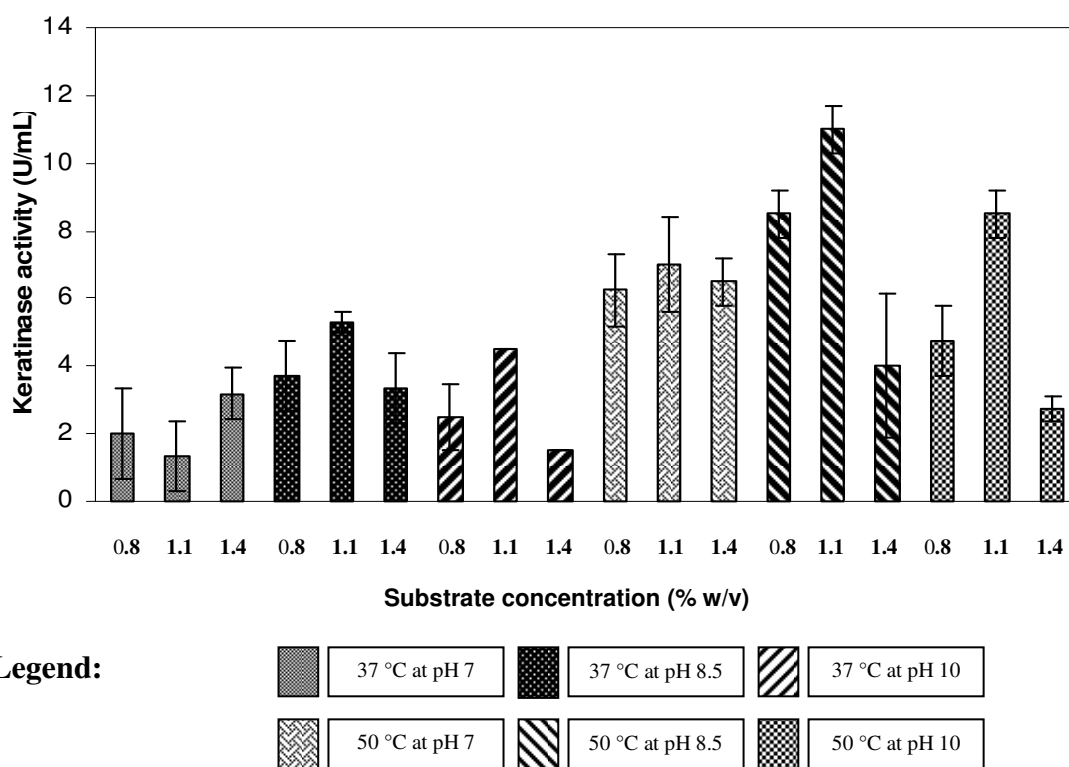


Fig. 3.10: Optimisation design for determination of optimum conditions for keratinase production for *Bacillus licheniformis* N22. Optimum production condition was achieved at pH 8.5, Temperature 50 °C and substrate concentration of 0.55 g/l (1.1 % w/v). Each bar represents mean \pm SD (n = 3, each data point).

3.1.7 Effects of chemical agents, biosurfactant and pH on keratinase activity

Chemical and biological agents could activate or inhibit keratinase activity, thus these properties are used to classify keratinases and to infer their stability under various conditions. The reducing agent dithiothreitol (DTT) significantly enhanced keratinase activity by 86 % more than with crude keratinase, 34 % by the detergent Triton X-100 and

12 % by EDTA. SDS showed an inhibitory effect on keratinase activity while biosurfactant slightly activated keratinase. These effects were measured relative to the crude keratinase activity (control) defined as 100 % (Table 3.3).

Table 3.3: Effect of chemical agents (Chelators, reducing agents and detergents) and biosurfactant on the activity of keratinase on keratin azure substrate (n = 3, each data point).

Agent	Activity (Mean \pm SD)
Crude keratinase	11.00 \pm 0.71
EDTA (5mM)	12.3 \pm 0.00
DTT (5mM)	20.47 \pm 4.56
Triton X-100 (0.1 %)	14.77 \pm 2.45
SDS (0.1 %)	10.10 \pm 1.91
Biosurfactant	12.00 \pm 0.50

In order to achieve optimum keratinase activity, determination of the optimum pH condition for keratinase was required. The optimum activity for crude keratinase was measured at pH condition of 8.5 (Fig.3.11).

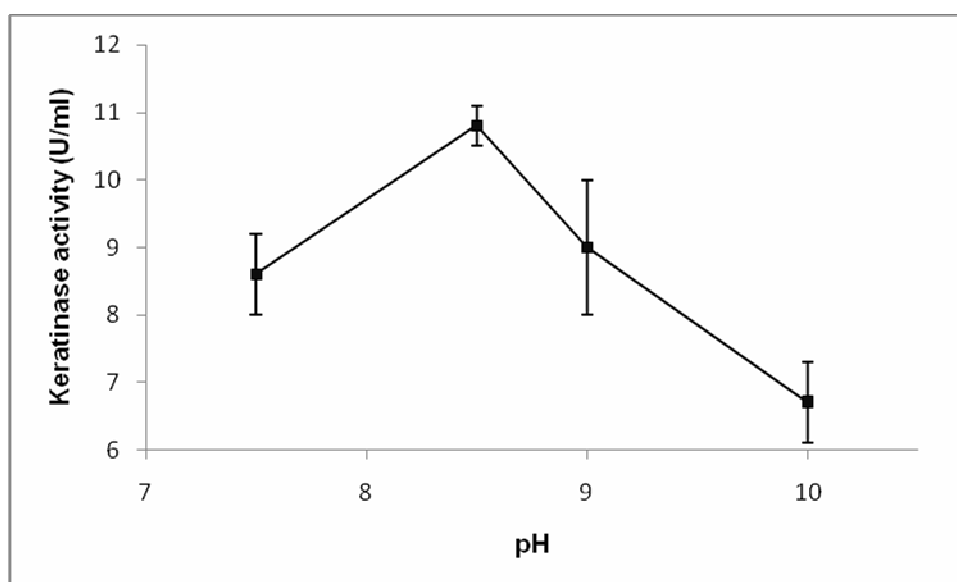


Fig. 3.11: Determination of optimum pH conditions for N22 crude keratinase in keratinase assay. Optimum keratinase was achieved at pH 8.5. Each data point represents mean \pm SD (n = 3, each data point).

3.1.8 Purification and characterisation of keratinase

Keratinase secreted in fermentation culture was recovered and purified to enable determination of its biochemical properties. Crude keratinase extract (120 ml) was concentrated down to 12.5 ml in a Centriconplus-70 centrifugal device of molecular weight cut-off of 10 KDa. Fractions from the purification steps along with the crude and concentrated crude enzyme extract are shown in Fig. 3.12. The keratinase activity, protein concentrations, specific activity, purification factor and percentage yield are presented in Table 3.4. The eluted fraction (EF2) of the second purification cycle had the highest specific activity (41.28 U/mg) and purification factor (1.97). The purified keratinase remained thermally stable, maintaining its activity for up to 8 weeks when stored in the fridge at 4 °C.

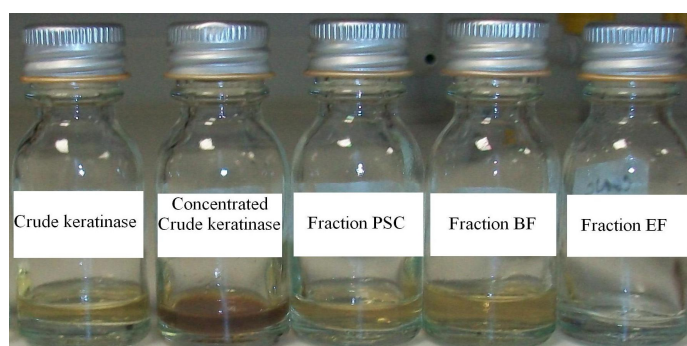


Fig. 3.12: Pooled fractions of the crude keratinase extract, concentrated crude keratinase preparation and fractions PSC, BF and EF derived from the purification steps of the step-wise feedback purification model.

Table 3.4: keratinase activity of crude keratinase and fractions from purification procedure, where, BF: fraction obtained with binding buffer; EF: fraction obtained with elution buffer; PSC: fraction obtained when sample is loaded; suffix numerals: number of cycles.

Fractions	Activity (U/ml)	Protein conc.(mg/ml)	Specific Activity (U/mg)	Purification factor	Yield (%)
Crude keratinase	10.5	0.502	20.91	1	100
Conc. crude keratinase	23	0.786	29.26	-	-
PSC1	17	0.839	20.26	0.98	161.84
BF1	19.3	0.820	23.53	1.13	183.74
EF1	3.5	0.128	27.34	1.31	33.32
PSC 2	20	0.557	35.90	1.72	190.4
BF2	11	0.368	29.89	1.43	104.72
EF2	4.5	0.109	41.28	1.97	42.84
PSC3	20	0.566	35.33	1.69	190.4
BF3	15	0.387	38.75	1.85	142.8
EF3	2	0.104	19.23	0.92	19.04

3.1.8.1 Molecular weight of purified keratinase

The molecular weight of purified keratinase was determined as approximately 28 KDa by both SDS-PAGE (on a 12 % gel) and MALDI TOF MS, and appeared as a single band on gel and a single peak in mass spectrum respectively (Fig. 3.13 and 3.14). No band was detected in the lane loaded with loading buffer (control). Single molecular weight band and mass peak of N22 keratinase were detected by SDS-PAGE and MALDI TOF MS respectively.

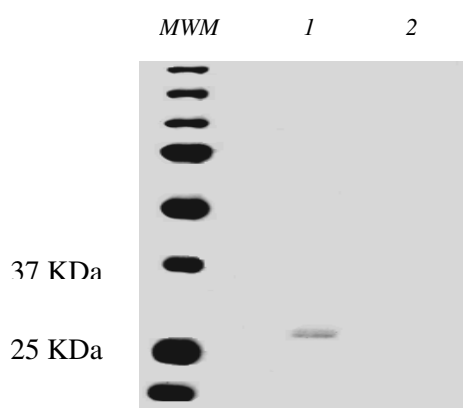


Fig. 3.13: Molecular weight band of keratinase at ≈ 28 KDa determined by SDS-PAGE (Lane 1) and matched against molecular weight marker (MWM) showing the 25 and 37 KDa bands. Lane 2 contained loading buffer (control).

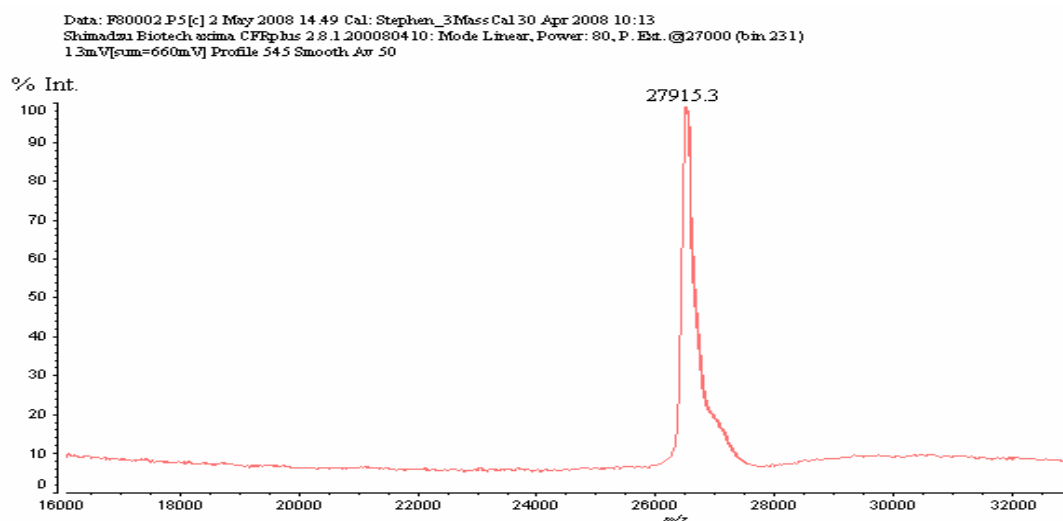


Fig. 3.14: MALDI-TOF MS spectrum showing molecular weight of keratinase as ≈ 28 KDa. Single peak depicts homogeneity of the sample.

3.1.8.2 Peptide mass fingerprint of keratinase

In order to establish peptide mass fingerprint for keratinase useful for its identification and characterisation, keratinase was digested for 2 h with 0.1 mg/ml of trypsin to generate tryptic peptides mass spectrum which was detected by MALDI-TOF MS (Fig.3.15 A-C). The original peak of ≈ 28 KDa was cleaved to generate a number of tryptic peptides. An

3.1.9 Characterisation of crude biosurfactant

To determine the biosurfactant concentration and total protein concentration, *Pseudomonas aeruginosa* NCIMB 8626 culture was ultra-filtered to obtain crude biosurfactant extract (Fig. 3.16). The concentration of crude biosurfactant extract was determined by the Orcinol method as 0.49 mg/ml (rhamnose equivalent) and the total protein concentration was measured as 0.72 mg/ml (BSA equivalent) by the Bradford reagent method. The pH of crude biosurfactant was measured as approximately pH 8.3. Crude biosurfactants remained effective when stored at 4 °C for more than a year.

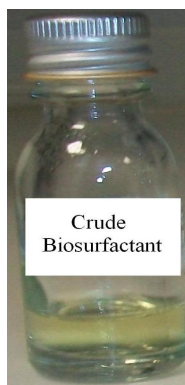


Fig. 3.16: Crude biosurfactant extract produced from *Pseudomonas aeruginosa* NCIMB 8626.

3.2 Degradation of keratin substrates and scrapie prions by keratinase

3.2.1 Degradation of keratin azure

Degradation of keratin azure substrate by keratinases results in the release of azure dye (blue dye) into reaction solution. To determine the keratinolytic characteristic of keratinase, keratin azure was digested with keratinase at 50 °C for 24 h resulting in significant release of azure dye (Fig. 3.17). In the control experiment (treated with denatured keratinase), minimal amount of blue dye was released.

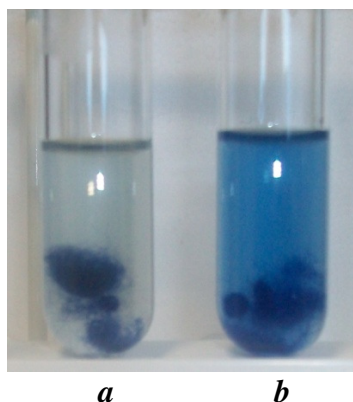
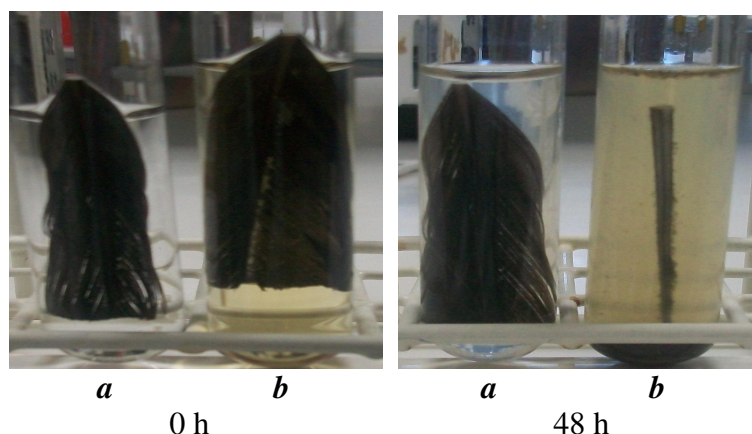


Fig. 3.17: Degradation of keratin azure by crude keratinase showing release of azure dye following cleavage of peptide derivatives. Test tube (a) was treated with boiled crude keratinase (Control) and test-tube (b) with intact (non-denatured) crude keratinase.

3.2.2 Degradation of melanised feather

Melanised feather are highly resistant to enzymatic degradation and the melanin pigment able to inactivate keratinase, so that only keratinases of high activity and stability are able to degrade melanised feather. To confirm that the feathers were free from microbial contamination which may aid degradation, the wash effluent was plated on nutrient agar plate and lacked microbial growth. To investigate the feather degrading ability of keratinase, melanised feathers was incubated with crude keratinase extract. The feathers were completely disintegrated and the mean feather weight was reduced from 52 ± 2 mg to 10 ± 3 mg in 48 h (Fig. 3.18). Feather incubated with distilled water only (negative control) showed no visual sign of degradation and no change in dry weight of feather. In contrast, denatured crude keratinase reduced the dry weight of the feather from 52 ± 2 mg to 46 ± 3 mg.

(A)



(B)

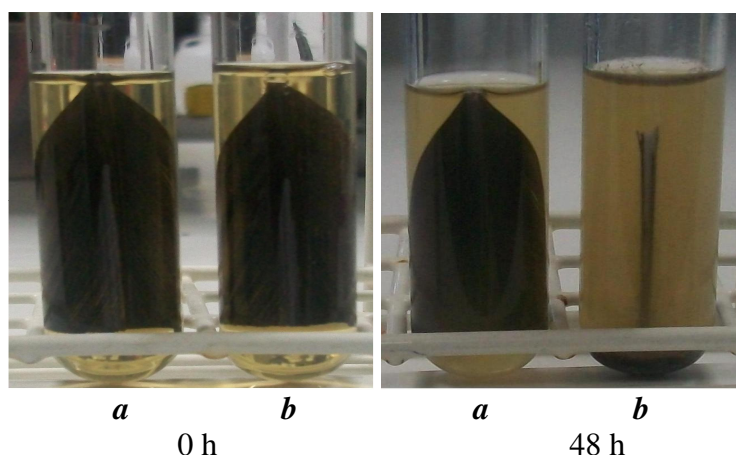


Fig. 3.18: Degradation of melanised feather by crude keratinase showing the state of feather at 0 and 48 h. In photographs labelled (A), the feathers in test tubes 'a' and 'b' were incubated in 10 ml of distilled water and dilute crude keratinase (2 ml in 8 ml of distilled water) respectively. In photographs labelled (B), the feathers in test tubes 'a' and 'b' were incubated in 10 ml of denatured crude keratinase (boiled for 30 min) and undiluted crude keratinase respectively.

3.2.3 Optimisation of western blot

Optimisation of western blot enables determination of procedural conditions and suitable materials such as membrane blocking buffer and membrane blocking duration that will produce and/or prevent cross-reactions, non-specific binding and unnecessarily long blocking duration. Keratinase (EF) cross-reacted with secondary antibody (anti-mouse IgG from sheep) when keratinase-bound PVDF membrane was blocked with 5 % BSA. This is shown by the detection of the molecular weight band of keratinase (≈ 28 KDa) on Western blot (Fig. 3.19, Lane 2). However, this cross-reaction was eliminated when 5 % skimmed milk was used as the blocking buffer (Fig. 3.19, Lane 1). The primary antibody (SAF83) did not cross react with EF. Furthermore, when the membrane blocking step was skipped altogether, no detectable background noise or non-specific binding was observed on the Western blot profile.

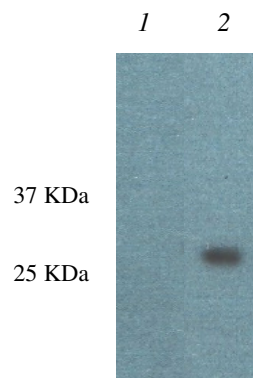


Fig. 3.19: Immunoreactivity of keratinase with secondary antibody (anti-mouse IgG from sheep) was absent when keratinase bound PVDF membrane was blocked with skimmed milk (Lane 1). Blocking with BSA resulted in immunoreaction indicated by the band at ≈ 28 KDa which is the molecular weight of keratinase.

3.2.4 Degradation of prion protein

PK completely degrades PrP^{C} and partially degrades PrP^{Sc} . To determine the prion degrading efficacy of keratinase (EF), PrP^{C} and PrP^{Sc} were digested with EF.

3.2.4.1 Degradation of normal prion protein (PrP^{C})

To determine the ability of EF to degrade PrP^{C} , normal brain homogenate (NBH) PrP^{C} was digested with EF at 50 °C for 1 h. PrP^{C} was completely degraded to a level undetectable by Western Blot analysis (Fig.3.20). Similarly, PrP^{C} was completely digested by PK but the sample that was not PK-digested (CNBH) remained undegraded (Fig.3.20).

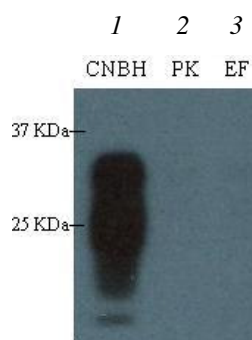


Fig. 3.20: PrP^C of normal brain homogenate (1 %) was proteolysed by PK (final conc. = 77 µg/ml) (Lane 2) and EF (lane 3) to undetectable levels whereas the control experiment remained intact. Lane 1 is undigested normal brain homogenate (positive control). PrP^C was probed with SAF83 mAb.

3.2.4.2 Degradation of scrapie prion

To examine the degradation of ME7 scrapie by keratinase, and to determine the keratinase purification fraction with the most prion-degrading activity, ME7 scrapie prion infected brain homogenate (1 %) was digested with crude keratinase extract (S), concentrated crude keratinase (CS) and PSC, BF and EF (fractions from the keratinase purification procedure [section 2.9.1]) and PK at 50 °C for 2 h. Western blot profile (fig. 3.21) show the order of increasing effectiveness for scrapie degradation as: S, CS, PSC, BF and EF. EF (purified keratinase) digested scrapie prion to levels of PrP^{Sc} undetectable by Western blot (Lane 8). PrPres remained after digestion with PK and the PrP signal of undigested scrapie positive control sample (CIBH) (Lane 1) remained fully detectable.



Fig. 3.21: Lane 1 is neat ME7 scrapie BH (positive control) and lane 2 is empty (negative control). Lane 3 is PK treated sample (final PK conc. = 77 µg/ml). Lanes 4 and 5 are samples digested with crude keratinase(S) and concentrated crude keratinase (CS) respectively. The fractions PSC, BF and EF (Lanes 5, 6 and 7 respectively) are described in the step-wise purification model. Digestion was carried out at 50 °C for 2 h. PrP^{Sc} was probed with SAF83 mAb.

3.2.4.3 Optimisation of degradation conditions

To further improve degradation of ME7 scrapie prion by keratinase, a biosurfactant (BS) was included in the reaction mixture and the digestion temperature, incubation time and enzyme concentration investigated to determine the optimum conditions for degrading ME7 scrapie with EF. ME7 scrapie digested with EF+BS enzymatic composition (1 μ l each) at 50 °C for 1 h resulted in the significant degradation of PrP^{Sc} relative to the other fractions, but trace levels of PrP^{Sc} signal remained detectable (Fig. 3.22; Lane 7). However, increasing the digestion time 2 h, EF+BS digested ME7 scrapie to undetectable levels (Fig. 3.23; Lane 3).

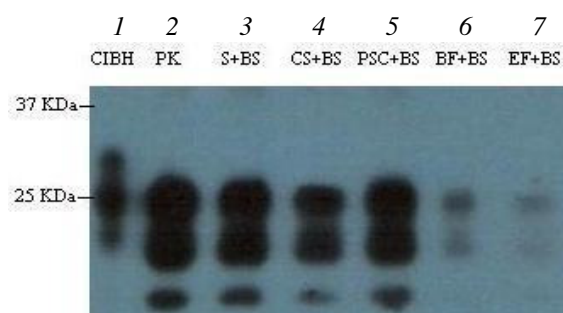


Fig. 3.22: Lane 1 is neat ME7 brain homogenate (positive control). Lane 2 is proteinase K (PK) treated sample (final PK conc. = 10 μ g/ml). Lanes 3 and 4 are ME7 brain homogenate digested with crude keratinase (S) and concentrated crude keratinase (CS) respectively. Lanes 6, 7 and 8 are digests with keratinase fractions PSC, BF and EF respectively. EF+BS digestion showed the most PrP^{Sc} signal removal. Digestion was carried out at 50 °C for 1h and PrP^{Sc} was probed with SAF83 mAb.

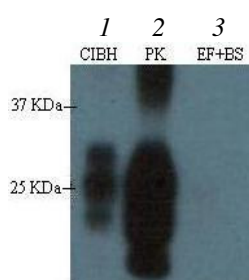


Fig. 3.23: Lane 1 is ME7 brain homogenate (positive control) and lane 2 is proteinase K treated sample (final PK conc. = 10 μ g/ml). Lane 3 is sample digested with EF+BS composition EF: BS (1:1). Samples were digested at 50 °C for 2 h. PrP^{Sc} was probed with SAF83 mAb.

By increasing the incubation temperature to 65 °C, EF+BS digested ME7 scrapie brain homogenate (10 μ l) in 1 h to undetectable level of PrP^{Sc} (Fig. 3.24). Under these conditions, EF alone was unable to completely degrade ME7 scrapie, resulting in typical

PrP^{Sc} glycosylation bands (Fig. 3.24; Lane 5). BS alone showed no discernible activity towards PrP^{Sc} degradation (Fig. 3.24; Lane 4).

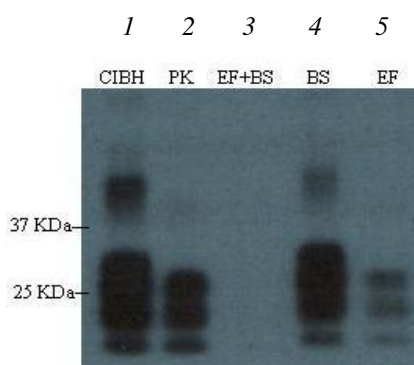


Fig. 3.24: Lane 1 is neat ME7 brain homogenate (positive control). ME7 scrapie infected brain homogenate digested at 65 °C for 1 h with PK (100 µg/ml), EF+BS composition, BS and EF (Lanes 2, 3, 4, and 5 respectively). Total removal of PrP^{Sc} signal was achieved with EF+BS but not with EF and BS working individually. PrP^{Sc} was probed with SAF83 mAb.

Furthermore, digestion of scrapie prion with the enzymatic composition (EF+BS) at 50 °C over a time-course of 30, 45, 60, 90, and 120 min, resulted in the gradual reduction of detectable PrP^{Sc} signal intensity over time (Fig. 3.25), with barely detectable levels at 120 min (Fig. 3.25; Lane 7). The diglycosylated (upper) band showed greater resistant to PrP^{Sc} degradation over the digestion period. Further optimisation showed a remarkably complete degradation of PrP^{Sc} to undetectable levels in 10 min (Fig. 3.26). PK also demonstrated a higher PrP^{Sc} digestion at 65 °C more than at 50 °C as shown by the PrP^{Sc} signal intensity in the lane with PK digested sample.

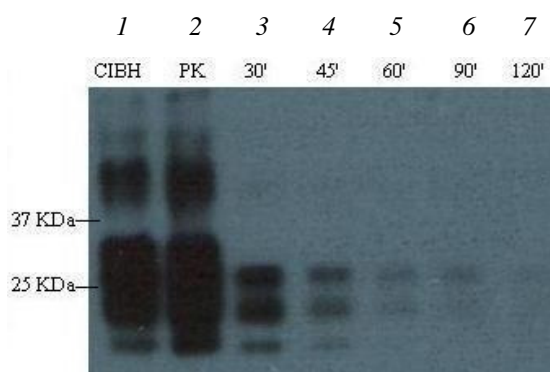


Fig. 3.25: Lane 1 is neat ME7 brain homogenate (positive control) and lane 2 is proteinase K treated sample (77 µg/ml final PK conc.). Lanes 3-7 are digested with EF +BS at 30-120 min respectively. Samples were digested at 50 °C with EF: BS ratio of 1:2. PrP^{Sc} was probed with SAF83 mAb.

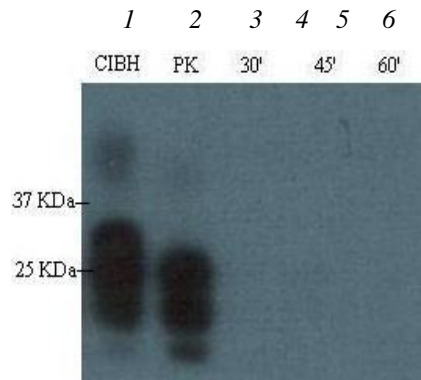


Fig. 3.26: Lane 1 is neat ME7 brain homogenate (positive control) and lane 2 is proteinase K digested sample (77 µg/ml final PK conc.). Lanes 3-6 are digested with EF+BS at 10, 30, 45 and 60 min. Samples were digested at 65 °C and PrP^{Sc} was probed with SAF83 mAb.

Normal brain homogenate (NBH) was completely digested by PK at concentrations of 10, 50 and 100 µg/ml (Fig. 3.27). ME7 scrapie brain homogenate (IBH) digested with similar concentrations of PK remain highly resistant to degradation, having only a slight band shift compared to the control and a marginal change in PrP^{Sc} intensity over the PK concentrations 10-100 µg/ml (Fig. 3.27). Samples digested with the enzymatic composition (EF+BS) at 1:50, 1:100 and 1:200 dilutions of the EF composite resulted in complete or nearly complete loss of PrP^{Sc} signal in order of increasing dilution (Fig. 3.27) which suggests a correlation between loss of PrP^{Sc} signal and concentration of EF.

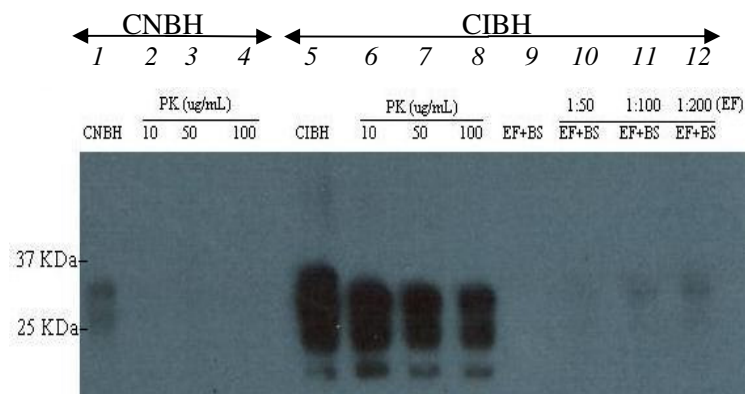


Fig. 3.27: Normal (CNBH) and infected (CIBH) brain homogenates digested at 65 °C for 1h with 10, 50 and 100 µg/ml of proteinase K (lanes 2, 3, 4 and lanes 6, 7, 8 respectively), and EF+BS composition with 1:50, 1:100 and 1:200 dilutions of EF (lanes 9, 10, 11, 12) probed with SAF83 monoclonal antibody. Lanes 1 and 5 are undigested NBH and IBH respectively.

3.2.4.4 Immunodetection with various monoclonal antibodies (mAbs)

To determine the monoclonal antibody (mAb) with high affinity for ME7 scrapie PrP^{Sc} and to ensure that no linear epitopes were missed out during immunodetection, enzyme digested ME7 scrapie PrP^{Sc} was immunodetected with various antibodies: SAF83, 98A3, 12B2, 9A2 and 6C2 (Section 2.12.1.2). Western blot (Fig. 3.28) show that SAF83 and 9A2 significantly detected PrP^{Sc} with high affinity, and mAbs 98A3 and 12B2 detected PrP^{Sc} slightly but not at all by 6C2. Residual PrP^{Sc} was not detected in EF+BS-digested materials probed with the various antibodies (Fig. 3.28).

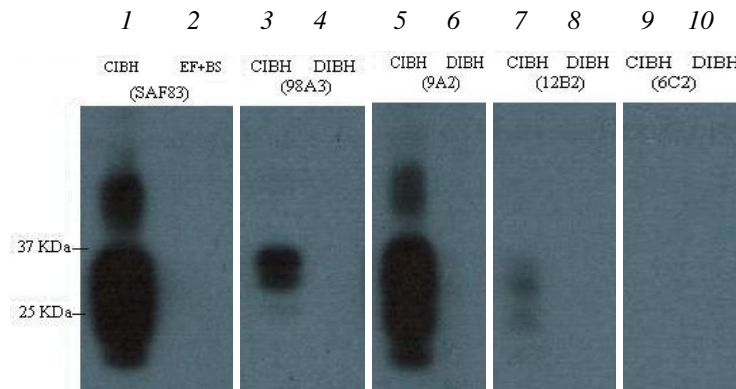


Fig. 3.28: ME7 scrapie prion digested with EF+BS at 65 °C for 1 h and PrP^{Sc} probed with SAF83, 98A3, 9A2 and 6C2. Lanes 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10 were probed with SAF83, 98A3, 9A2 and 6C2 respectively. Lanes 1, 3, 5, 7 and 9 are the corresponding positive controls.

3.3 Residual infectivity evaluation of keratinase-digested scrapie prions

3.3.1 Scrapie cell assay

To investigate the efficacy of the enzymatic composition, EF+BS, to completely destroy prion infectivity, and to confirm if the complete loss of PrP^{Sc} signal as detected by Western blot correlated loss prion infectivity, SSBP/1 susceptible Rov9 cells were inoculated with enzyme-digested SSBP/1 scrapie and analysed for residual infectivity. SSBP/1 was used for the scrapie cell assay as ME7 scrapie is not susceptible to Rov9 cells and no ME7 susceptible cells were available. ME7 and SSBP/1 (along with other scrapie strains) were similarly degraded to undetectable levels by EF+BS as determined by Western Blotting (appendix 4), hence their degradation by EF+BS were considered to be comparable. Immunodetection of infected Rov9 cells by ELISPOT assay confirmed that Rov9 cells propagated SSBP/1 scrapie prion but Rov9 cells inoculated with EF and EF+BS digested SSBP/1 scrapie did not propagate infectivity as shown by the absence of infected cells (detectable spots) in the ELISPOT assay (Fig. 3.29).

The number of infected cells (spots detected) per 5000 cells (in each well as confirmed by trypan blue assay) were significantly lower for both the EF and EF+BS treatment groups compared to the incubated IBH group ($p = 0.009$, Two-Sample T-Test; Fig. 3.30). Similarly, a significantly lower number of cells were infected in the BS treatment group compared to the incubated IBH group ($p = 0.033$, Two-Sample T-Test; Fig. 3.30) for BS treatment group, but was significant to propagate infection in Rov9 cells. Furthermore, SSBP/1 infectivity was significantly reduced by incubation at 65 °C as determined by the spots detected for incubated IBH compared to IBH ($p = 0.044$, Two-Sample T-Test; Fig. 3.30). All experiments were repeated in quadruplet and the number of positive cells (spots) were counted using Zeiss KS-ELISPOT imaging system running Wellscan software.

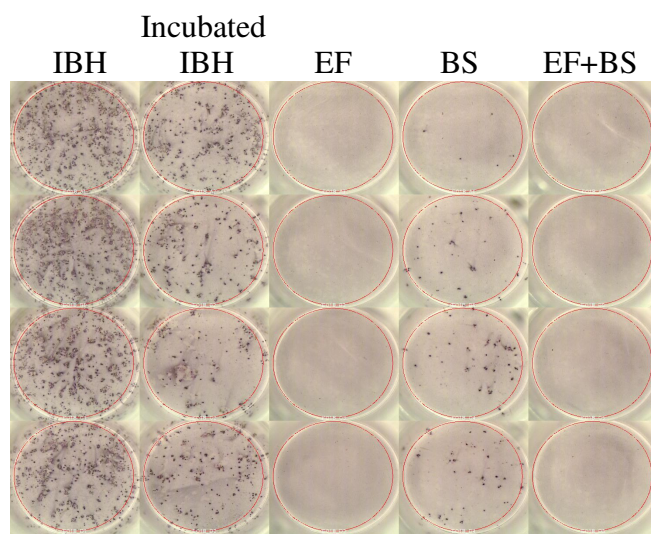


Fig. 3.29: Photograph of ELISPOT plate acquired with Zeiss KS-ELISPOT imaging system showing infected Rov9 cells in wells inoculated with IBH (SSBP/1), incubated IBH (heat treated SSBP/1) and BS (Biosurfactant digested SSBP/1). Wells inoculated with SSBP/1 digested with EF and EF+BS were completely devoid of infected cells. PrP^{Sc} was probed with sha31 mAb.

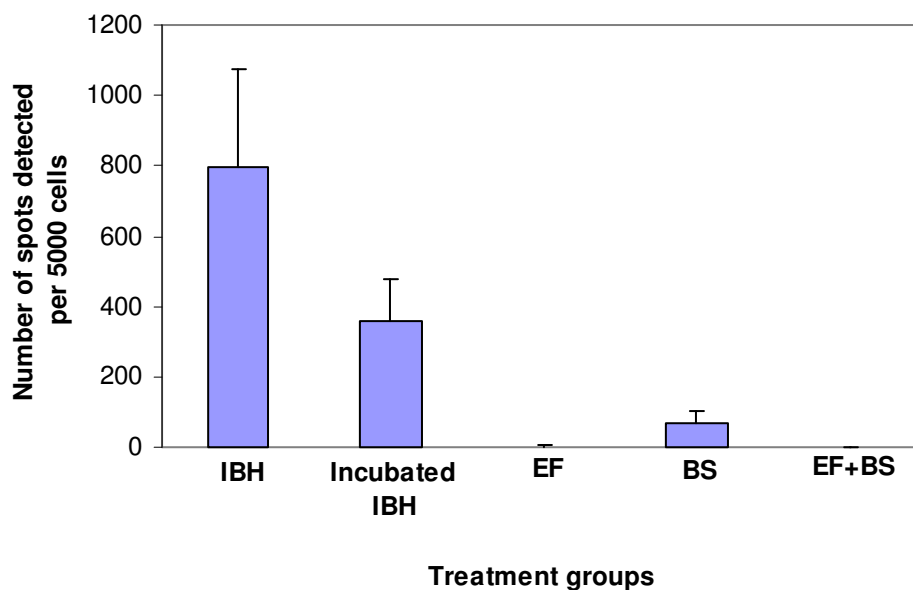


Fig. 3.30: Number of infected cells (spots) detected by Zeiss KS-ELISPOT imaging system for the different treatment groups; IBH (inoculated with neat SSBP/1), Incubated IBH (inoculated with SSBP/1 that was incubated at the digestion temperature), EF (inoculated with keratinase, EF), BS (inoculated with biosurfactant, BS) and EF+BS (inoculated with enzymatic composition, EF+BS). The number of infected cells were significantly reduced for EF ($p = 0.009$), BS ($p = 0.033$) and EF+BS ($p = 0.009$) treatment groups compared to Incubated IBH. Test of significance was calculated using Two-Sample T-Test. Each bar represents mean \pm SD ($n = 4$, each data point).

3.3.2 Mouse bioassay

In order to investigate whether the loss of PrP^{Sc} signal seen in *in vitro* Western blot was accompanied by a corresponding loss in prion infectivity, *in vivo* mouse bioassay was performed. The result of the bioassay is summarised in Table 3.5. Brain samples of culled/dead mice were homogenised in sterile PBS and analysed for residual PrP^{Sc} by Western blotting after PK digestion. The electrophoretic profiles are presented in appendix 1.

Table 3.5: Summary of mouse bioassay showing the different treatment groups (treated and control groups) and the number of mice challenged, number found dead/culled, number with detectable PrP^{Sc}, survival time range and mean survival time for PrP^{Sc} positive mice.

Treatment Group	Individual Survival Times (days) ^a	PrP ^{Sc} in Brain	Incidence ^c (% Incidence)	^d Mean Survival Time (days \pm SD) or (Survival Range)
NBH (Digested) (-ve control)	384, 496	No	0/2 (0 %)	ND
IBH (Neat) +ve control)	275, 275, 275, 269, 276, 283, 296	Yes	7/7 (100 %)	278 \pm 9 (269 - 380)
IBH (1:10 dilution) (+ve control)	204, 217, 259, 303, 321	Yes	5/5 (100 %)	261 \pm 52 (204 - 321)
IBH (1:100 dilution) (+ve control)	328, 334, 334, 336, 340, 343, 345, 369	Yes	8/8 (100 %)	341 \pm 13 (328 - 369)
IBH (1:1000 dilution) (+ve control)	> 550	ND ^b	ND	ND
IBH (Digested)	181, 186, 191, 191, 233, 233, 243	No	8/15 (53 %)	334 \pm 42 (296 - 431)
	296, 299, 321, 325, 327, 332, 342, 431	Yes		

^a Only mice that were culled or found dead at \leq 550 days were included in the analysis.

^b Animals survived longer than 550 days and their PrP^{Sc} status not determined (ND)

^c Incidence = number of animals affected/number of animals tested.

^d Only mice that were PrP^{Sc} positive were included in the survival time and range.

Three (3) mice (60 %), 4 mice (44 %), 5 mice (38 %) and 6 mice (100 %) in the NBH digested, IBH 1:10, 1:100 and 1:1000 dilutions treatment groups respectively, survived beyond the termination of the experiment 550 days (see appendix 2). All the surviving mice were not tested for the presence of PrP^{Sc} in their brains and were not included in the survival time analysis to allow for direct comparison with IBH Neat (positive control) group. Similarly, all PrP^{Sc} negative culled/dead mice were not included in the analysis. The

Western blot analysis of dead/culled mice brain homogenates from the positive control groups (IBH Neat, IBH 1:10 and 1:100 dilutions of inocula) showed that all the mice tested positive for PrP^{Sc} (100 % disease incidence) whereas no PrP^{Sc} were found in those of the NBH digested group (negative control). Eight (8) mice were PrP^{Sc} positive in the IBH-digested group (53 % disease incidence) with survival time post-inoculation ≥ 296 days (mean survival time = 334 ± 42 days), whereas all seven mice (47 % of inoculated mice in this group) that tested PrP^{Sc} negative had a survival time ≤ 243 days (mean survival time = 208 ± 27 days) post-inoculation. A significant difference existed between the two sub-populations in the IBH-digested group ($p = 0.000$, Two-Sample T-Test). There was a significant difference in the incubation times for the PrP^{Sc} negative mice in the IBH-digested group compared to mice in the IBH neat (positive control) group ($p = 0.000$, Two-Sample T-Test). The mean survival times for IBH (neat), 1:10 dilution, 1:100 dilutions and IBH (digested) groups were 278 ± 9 , 261 ± 51 , 341 ± 13 , and 334 ± 42 days respectively. Significant difference in survival times existed for the treatment groups ($p = 0.000$, One-way ANOVA). Furthermore, a significant increase in survival time ($p = 0.008$, Two-Sample T-Test) was found between IBH (digested) group and IBH neat group (positive control).

Boxplot of the survival times for the treatment groups showing the individual and mean survival times for each treatment group is presented in Fig. 3.31.

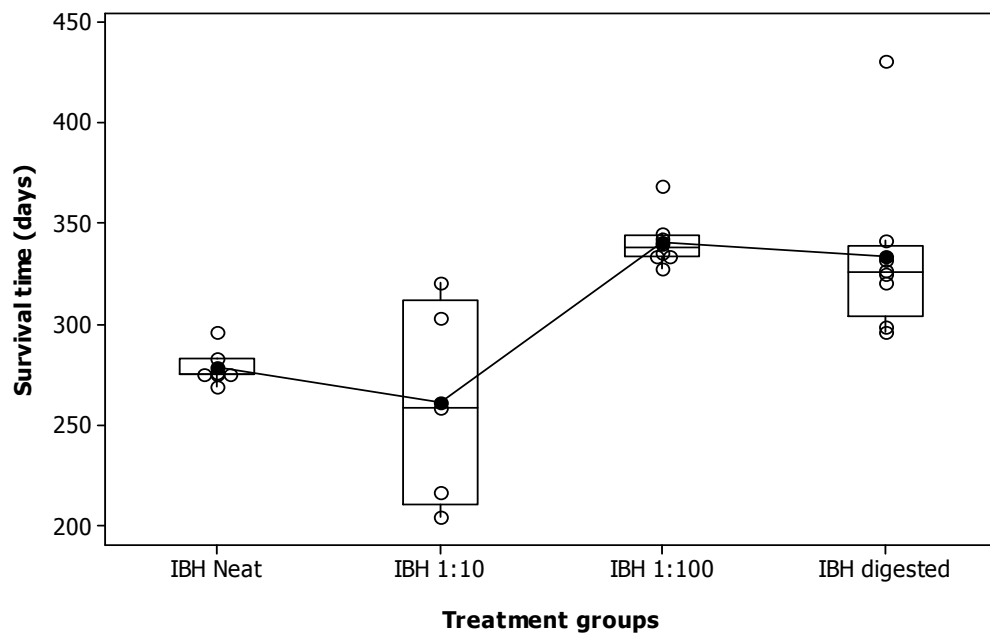


Fig. 3.31: Boxplot showing the survival times of infected (PrP^{Sc} positive) mice in the different treatment groups: IBH Neat, IBH 1:10 dilution, IBH 1:100 dilution and IBH digested. Survival times for individual mice are represented by the circles and circle with black fill is the mean survival times linked by the mean connect line.

3.3.2.1 *PrP^C expression in wild type and Tga20 mice used in bioassay*

Due to the long and dispersed survival times within and between the treatment groups of the bioassay (appendix 2), the levels of PrP^C of the Tga20 test mice were compared with wild-type Charles River C57BL/6 mice as analysed by Western Blotting. The electrophoretic profile (Fig. 3.32) showed variable levels of PrP^C in the Tga20 brain homogenate sample (265, 271, 291 and 281), and were generally comparable to the wild-type mice.

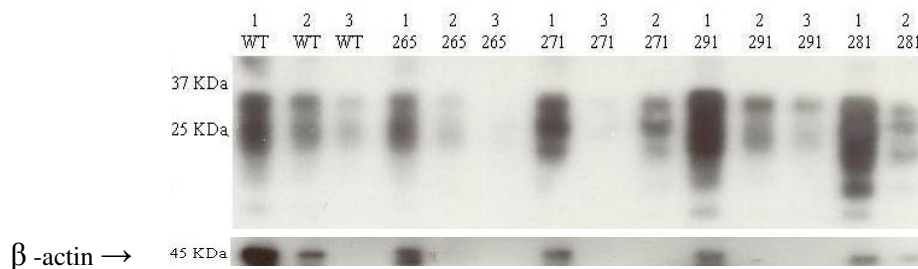


Fig. 3.32: Test of PrP^C levels in brain homogenates samples of Charles River C57BL/6 wild type mice and the Tga20 mice used for the bioassay. 1, 2 and 3 were undiluted, 1/3 dilutions and 1/9 dilutions respectively. WT were Charles River C57BL/6 and 265, 271, 291 and 281 were Tga20 mice samples. PrP^C was probed with SAF83 mAb.

Chapter 4: Discussion

4.1 Research overview

Prions, the agent implicated in transmissible spongiform encephalopathies or prion diseases, are highly resistant to degradation by conventional sterilisation and inactivation method. Physical and chemical methods such as incineration and alkaline hydrolysis known to degrade prions are not compatible for use in many applications such as those that will require recovery of contaminated materials and those that are destroyed by corrosion. To overcome these limitations, enzymatic methods for prion degradation have become increasingly important.

In this study, the enzymatic degradation of pathogenic prion protein by keratinase producing microorganisms was investigated. Microorganisms were isolated from farmyard wastes and primary effluent known to be rich sources of microorganisms and extracellular proteases (Semple *et al*, 2001; Torks *et al*, 2010). Prions are structurally similar to feather keratins in terms of their high β -sheet composition, which accords them with mechanical stability, insolubility and resistance to degradation by most proteases. Because some microorganisms are able to degrade feathers, feather meal agar was used for isolation of potential prion degrading bacteria. The isolates were further tested for proteolytic activity using casein-agar assays for the reason that casein is a highly stable substrate degraded only by proteases of high enzymatic activity. Furthermore, definitive test of keratinolytic activity was performed by keratinase assay using keratin azure as substrate. The keratinase assay was standardised and validated as a reliable test of keratinolysis. A highly proteolytic bacterium, designated *Bacillus licheniformis* N22, was identified and characterised, and the optimum temperature, pH and substrate concentration conditions for fermentation production of keratinase determined. Crude keratinase extract was recovered, purified and characterised by SDS-PAGE and MALDI-TOF MS to determine the molecular weight, purity and peptide mass fingerprint of the keratinase. The thermal stability and effects of chemical agents on the keratinase activity were also determined. The N22 keratinase was further evaluated for keratinolytic ability on melanised feather; the melanin of which confers higher resistance to degradation by keratinases compared to white feathers, and coupled with the β -sheet content of feather keratin, makes it a suitable substrate for testing potential prion-degrading keratinases. N22 keratinase completely degraded melanised feather and also degraded ME7 scrapie to levels undetectable by Western Blot analysis. Biosurfactant produced from *Pseudomonas aeruginosa* NCIMB 8626 was used to enhance degradation of scrapie prion. Test of residual infectivity by standard scrapie cell assay confirmed that complete loss of PrP^{Sc} signal in the Western Blot analysis was related to

loss of prion infectivity, although the result of mouse bioassay was not definitive. Overall, the results discussed in the following sections suggest that the experimental strategy has been successful in achieving the central aim of this research.

4.2 Isolation and characterisation of proteolytic microorganisms, keratinase and biosurfactant

4.2.1 Screening methods for proteolytic microorganisms

The casein-agar assays are useful preliminary methods for detection of proteolysis, and are efficient, cost and labour effective for rapid and bulk screening as well as characterisation of proteolytic microorganisms and their proteases (Brandelli and Riffel, 2005, Joshi *et al*, 2007; Lin *et al*, 1992; Olaguyigbe and Ajele, 2005). Casein is a highly stable, hydrophobic and an extremely slow-digesting non-fibrous protein, which is not coagulated or easily denatured by heat but is hydrolysed by certain proteases of high proteolytic activity (Fujiwara *et al*, 1993; Takami *et al*, 1992; Cheng *et al*, 1995). This characteristic was used in this study as a criterion for screening proteolytic microorganisms that are able to hydrolyse casein and similar structurally stable substrates such as keratins (Fig. 3.1). The formation of casein hydrolysis ring on casein-agar plates characterised proteolysis and was used for identifying proteolytic microorganisms. The size of the casein hydrolysis rings was used to compare proteolytic activity. A correlation of hydrolysis ring size and protease concentration was demonstrated (Fig. 3.2), which establishes the hydrolysis ring assay as a semi-quantitative method for measuring proteolytic activities and comparing proteolytic microorganisms. In addition to identifying proteolytic activity, the spot inoculation method was used for microbial characterisation in the determination of optimum temperature condition for cell growth and protease synthesis (Fig. 3.7). The hydrolysis ring assay enables the characterisation of extracellularly synthesised protease extracts, and the screening of purification fractions for proteolytic activity. However, since not all proteases are likely to be keratinolytic, evaluation of keratinolytic activity and identification of potential keratinase producers was performed with keratinase assay, which ideally utilises keratin azure as substrate and hence is highly specific for determination of keratinase activity.

Comparing keratinase activity of the same or different keratinase producing microorganism within and across laboratories has been rendered impossible by variable assays for evaluating keratinase activity. The need for a standard assay that will enable quantitative comparison of keratinase activity has been previously suggested (Scott and Untereiner, 2004; Gradisar *et al*, 2005), but until now no attempts have been made to address this problem. A reproducible and reliable keratinase activity measurement depends on a

number of critical factors such as homogeneity of test substrate and consistency of assay procedure. The commonly used assay for keratinase activity measurement (keratinase assay) uses keratin azure (a blue fibrous compound made by impregnating sheep wool keratin with azure dye) as substrate (Apodaca and McKerrow, 1990; Letourneau *et al*, 1997; Tadeusz, 1999; Bressollier *et al*, 1999; Scott and Untereiner, 2004; Esawy, 2007; Tapia and Simoes, 2008).

Keratin azure is considered as a suitable substrate for keratinase assay because the intensity of azure-bound amino acids or peptides released into solution by proteolytic cleavage of keratin bonds is easily measured by spectrophotometry. It is highly resistant to denaturation and degradation by common proteases, but more specifically degraded by keratinases. However, it has been reported for the first time in this study that the dye distribution of commercial keratin azure (K8500) substrate is heterogeneous, resulting in inconsistent, irreproducible and unreliable measurements between sample replicates. This is evidenced by variable release of azure dye into reaction mixture as measured by spectrophotometry. In order to address this problem, commercial keratin azure was standardised prior to use in keratinase assay by picking out solid azure particles, and incubating and washing with distilled water. This procedure removed loosely-bound azure dye and dye particles adhering to the keratin fibres such that the resulting keratin azure became homogenous (Table 3.1). Thus, the dye released into the reaction mixture during keratinase assay was entirely as a consequence of keratin azure hydrolysis.

Standardisation of commercial keratin azure to yield a homogeneous substrate (Table 3.1) enabled reliable comparison of keratinase activity and grading of keratinases according to their keratin degrading ability. As a model keratinaceous substrate, standardised keratin azure is important for assessing potential applications of keratinases for feather waste and hides processing and prion degradation. It will also enable comparison of keratinases in a more consistent manner across laboratories unlike the current practice of using heterogeneous keratin azure or different substrates types such as chicken feather (Anbu *et al*, 2007), azo-keratin (Lin *et al*, 1992) and keratin powder made from human sole (Gradisar *et al*, 2005).

The procedure for keratinase assay is simple, reliable and reproducible, and hence, can be used as the standard assay for keratinase activity measurement. However, in order to achieve the optimum activity for specific keratinases, their optimum reaction conditions are required. In this study, the keratinase assay was carried out at 50 °C and pH 8.5 for 1 h, which were adequate to catalyse keratinase reaction and to achieve optimum activity.

Under these conditions, the standardised keratin azure was generally stable such that azure dye released into the reaction mixture was solely as a result of keratinase activity. The optimum pH condition, for instance, was typical for most keratinases (approximately pH 8-8.5), which appears to be quite robust for the evaluation of the keratinase activity (Letourneau *et al*, 1998; Bressollier *et al*, 1999; Riffel and Brandelli, 2006; Gradisar *et al*, 2005).

4.2.2 Screening and selection of working isolate

Since the first report on the isolation of extracellular alkaline serine protease from *Bacillus* sp strain 221 (Horikoshi, 1971), the quest for industrially relevant proteases from different environmental sources has not relented. In the same vein, the central aim of this study was to isolate keratinase producing microorganisms of prion degrading ability from environmental sources such as farmyard wastes (e.g. chicken wastes) which is known to be a rich source of keratin degrading microbial populations (Tork *et al*, 2010), and sewage effluent which is an important source of extracellular proteases (Semple *et al*, 2001; Drouin *et al*, 2008). Since feather degrading microorganisms utilises feather keratin as a source of carbon and energy for growth and survival, potential keratin degrading microorganisms could be isolated on feather meal agar as have been previously reported (Brandelli and Riffel, 2005; Tapia and Simoes, 2008). A total of thirty-two microbial isolates were selected and differentiated based on their ability to grow on feather meal agar and their distinctive characteristic features. These isolates were screened for proteolytic and keratinolytic activity by the casein-agar plate assays and keratinase assay respectively. One isolate identified as isolate number 22 demonstrated the highest keratinase activity (Fig. 3.3) and was selected for further study.

Evaluation of the microbial isolates for potential prion degrading activity was performed using the proteinase K calibration curve which was established in this study to compare keratinases with PK for prion degrading potentials. The proteinase K calibration curve was plotted based on PK activity on keratin azure substrate (Fig. 3.4). PK was selected as the active protease because it is a keratinolytic protease of broad specificity and is commonly used for prion digestion and characterisation (Prusiner *et al*, 1983; McKinley *et al*, 1983; Pastrana *et al*, 2006). Keratin azure is highly stable against common proteases, and is the primary substrate of PK. Most importantly, keratins are structurally similar to prions in terms of their β -sheet content.

PK concentration at 0.2 mg/ml has been reported to fully degrade prion under denaturing conditions (preheated at 115 °C) to levels undetectable by Western blot analysis (Langeveld *et al*, 2003). Therefore, a reference PK activity cut-off on the PK calibration

curve at PK concentration of 0.2 mg/ml was adopted as minimum criteria for selecting keratinases of significant prion degrading activity. Keratinases of activities equal to or greater than the reference PK activity cut-off were considered to be potentially prion degrading, at least under denaturing conditions. By this criterion, about 68 % of the selected strains were adjudged to be potentially prion degrading, suggesting that the screening and isolation strategy for selection of potential prion degrading keratinolytic isolates was successful. The isolate number 22, significantly exceeded the PK activity reference cut-off by 54 %, and was selected for further investigation.

It is important to note that this criterion for selecting potentially useful prion degrading keratinases does not undermine the specificity and affinity of individual keratinases for prion degradation or their interactions in the presence of other agents. For example, it has been reported that the total proteolytic activity of an organism does not necessarily correlate with its ability to degrade PrP^{Sc} (Muller-Hellwig *et al*, 2006), which suggests that specific proteases are required for PrP^{Sc} degradation, although proteases of high proteolytic activity are potentially more likely to degrade PrP^{Sc} more efficiently. Therefore, confirmation of prion degrading ability is best carried out by degrading actual prion substrate. Furthermore, the PK calibration curve method for selecting potential prion degrading microorganisms provides an important start-off point, and has indeed proven to be useful in this study.

4.2.3 Identification and characterisation of isolate

The selected isolate number 22 was identified and designated *Bacillus licheniformis* N22 strain (section 3.1.5) and was deposited with the National Collection of Industrial Food and Marine Bacteria (NCIMB) with designated ascension number NCIMB 41708. *Bacillus licheniformis* N22 is a gram-positive, endospore-forming, mostly alkalophilic, mildly thermophilic and halotolerant bacterium (Table 3.1). This bacterium gave very strong catalase reaction as similarly reported for *Bacillus licheniformis* PWD-1 (William *et al*, 1990). Such strong catalase reaction suggests high levels of the membrane protein cytochrome oxidase which is important in pH homeostasis, and ensures the organism's survival over wide range of pH environments. The growth response at 50 °C was faster than growth at 37 °C, reaching visible growth on nutrient agar 4 h earlier than at 37 °C. *Bacillus licheniformis* N22 grows aerobically and at moderate temperature (37-60 °C) and pH (6-10) conditions. Hence it is attractive from the technological and practical point of view for the production of N22 keratinase and biotechnological applications in general.

The MALDI TOF-MS microbial mass spectrum acquired for *Bacillus licheniformis* N22 (Fig. 3.6) differed from those previously reported for other *Bacillus* species (Pusch and

Kostrzewa, 2006) and hence contributes to the developing database of this fast, robust and reliable microbial identification method. These microbial mass peaks found within the mass range of 2000-20000 Daltons represent mainly ribosomal proteins (Suh *et al*, 2005; Maier and Kostrzewa, 2007), and are sufficient to distinguish microbial strains (Pusch and Kostrzewa, 2006).

The prolific keratinase producing potential of *Bacillus licheniformis* N22 is characteristic of most *Bacillus licheniformis* species (Kitada *et al*, 1987; William *et al*, 1990; Manczinger *et al*, 2003). *Bacillus licheniformis* N22 differs from the other well characterised keratinase producing *Bacillus* such as *Bacillus licheniformis* PWD-1 (William *et al*, 1990) and *Bacillus* sp PW (Joshi *et al*, 2007). For example, *Bacillus licheniformis* PWD-1 produces acid from lactose but *Bacillus licheniformis* N22 does not, while *Bacillus* sp PW produces both acid and gas. These characteristics may represent important differences in their molecular constitution.

4.2.4 Optimisation of keratinase production

The interplay of various factors such as temperature, pH, substrate concentration, the nature and composition of carbon and nitrogen sources and the condition of inoculants, influences cell growth and survival, and the levels of protease synthesis and secretion in a microbial culture (Singh *et al*, 1975; North, 1982). Optimisation of these factors results in optimum conditions of microbial growth and synthesis of bioproducts. The optimum condition for the fermentation production of N22 keratinase was achieved at substrate concentration of 1.1 %, pH 8.5 and 50 °C over a fermentation time of 32 h. The strongest limiting effect on keratinase synthesis was induced by low pH condition ($\text{pH} \leq 4$), followed by high temperature (≥ 63 °C) condition.

The final pH of the fermentation culture as measured at the end of incubation time (32 h) was pH 8.3 irrespective of initial culture pH conditions (pH 6-12) (Fig. 3.9). A similar shift in pH from 7 to 8.5 has been reported for *Bacillus licheniformis* PWD-1 (William *et al*, 1990). The microbial cell interactions in the *Bacillus licheniformis* N22 fermentation culture resulting in a buffering effect and the culture pH tending toward pH 8.3 suggests at least two possible metabolic pathways depending on the prevailing culture condition. One pathway which is able to synthesise metabolic products that tend to up-regulate (acidic product) the culture pH, and the other which is able to synthesis metabolic products that down-regulate (alkaline products) the culture pH. A decreased culture pH during cultivation of *Bacillus* species has been attributed to the production of organic acids (Paavilainen *et al*, 1995). Formic acid has been reported to stabilise β -sheet structure (Aluigi *et al*, 2007). Considering that the feather meal substrate consists of keratin of high

β -sheet content, it is possible that destabilisation of β -sheet structure of feather keratin substrate results in the release of formic acid into the culture medium thereby increasing acidity. On the other hand, increased pH in fermentation cultures have been associated with the mechanism of microbial utilisation of protein substrates such as has been described for *Chryseobacterium* sp. Kr6 in feather meal culture (Riffel and Brandelli, 2006), and *Bacillus pumilus* grown in bovine hair culture (Kumar *et al*, 2008). Also, production of ammonia caused by the deamination peptides and amino acids during the degradation of keratin has been reported to increase culture pH (Kumar *et al*, 2008). Increases in fermentation culture pH have also been related to some level of keratinolytic activity (Kunert, 2000; Kaul and Sumbali, 1997). In general, one can argue that the regulatory mechanism that enables *Bacillus licheniformis* N22 to thrive at both alkaline and slightly acidic environment suggests wide utilitarian potential capacity for this bacterium.

The buffering pH level measured in the *Bacillus licheniformis* N22 fermentation culture medium closely mimics the optimal pH for keratinase synthesis which could suggest that setting the initial fermentation culture at the optimum pH would avert possible energy dissipation in establishing a physiological balance for a suitable culture environment for optimum production of keratinase. The pH kinetic plot (Fig. 3.9) developed during this study clearly defined the pH threshold for fermentation culture, identified the optimum pH level and the extreme pH conditions. For *Bacillus licheniformis* N22, the extreme pH conditions (pH < 5 or >12) were intolerable and hindered cell growth, survival and keratinase production (Fig. 3.8). Extreme pH affects cell physiology as it tries to achieve pH homeostasis and survival resulting in poor enzyme production (Kitada *et al*, 1987). The results of pH and temperature characterisation for *Bacillus licheniformis* N22 as determined by both casein-agar (Fig. 3.7) and keratinase assays (Fig. 3.8) were within the range of optimum conditions reported for some other keratinase producing microorganisms such as *Bacillus licheniformis* PWD-1 (pH 7- 8.5, 37 °C; Wang and Shih, 1999), *Bacillus licheniformis* K-508 (pH 7, 47 °C; Manczinger *et al*, 2003), *Streptomyces* (pH 8, 40 °C; Tapia and Simoes, 2008), *Bacillus species* (pH 8, 60 °C; Olajuyigbe, and Ajele, 2005) and *Streptomyces* strain BA₇ (pH 8.5, 50 °C; Korkmaz *et al*, 2003).

Keratin substrates such as feather meal and chicken feather induces keratinase production (Wang and Shih, 1999; Brandelli and Riffel, 2005; Thys *et al*, 2006; Letourneau *et al*, 1998; Chao *et al*, 2007; Anbu *et al*, 2007). Similarly, feather meal substrate induced keratinase synthesis by *Bacillus licheniformis* N22 resulting in 68 % increase in keratinase production compared to culture grown in nutrient broth medium. Generally, the optimum keratinase production was achieved at feather meal substrate concentration of 1.1 % for

various conditions of pH and temperature (Fig. 3.10) except for culture growing at pH 7 and 37 °C. Increasing the substrate concentration to 1.4 % resulted in reduced levels of keratinase production similar to previously reported repression of keratinase production at high substrate concentrations (Wang and Shih, 1999; Joo *et al*, 2002; Riffel *et al*, 2003a; Brandelli and Riffel, 2005; Cai *et al*, 2008; Lin and Yin, 2010). Growth substrates constitutes around 30-40 % of total production cost of enzymes (Kumar and Parrack, 2003). Therefore, the use of low cost and readily available keratin substrates such as feather meal or chicken feather will enable production of N22 keratinase at industrially relevant scale. Currently, billions of tonnes of chicken feather waste is produced annually (American Chemical Society, 2011), making it a potentially important raw material. In addition, the use of feather waste as growth substrates for keratinase production will invariably serve as an efficient way to manage the large amount of feather wastes produced in the poultry industry.

Crude keratinase extract of *Bacillus licheniformis* N22 expressed 58 % more activity on keratin azure substrate compared to the well characterised and industrially useful keratinase of *Bacillus licheniformis* PWD-1 (section 3.1.4). Recently, it was reported that *Bacillus* sp PW keratinase remarkably expressed 39 % more activity than *Bacillus licheniformis* PWD-1 (Joshi *et al*, 2007). Therefore, it can be argued that *Bacillus licheniformis* N22 keratinase has a relatively much higher keratinolytic activity than both keratinases.

4.2.5 Purification and characterisation of keratinase

The purification of N22 keratinase was performed by a step-wise feedback model (Fig. 2.2) executed over three purification cycles using commercially available HiTrap™ Blue HP column in which the column material consisted of resin/dye ligand complex to which keratinase adsorbed either biospecifically or less specifically by electrostatic and/or hydrophobic interaction. The step-wise feedback purification strategy enabled substantial recovery of purified keratinase over multiple purification cycles by reintroducing eluted excess crude extract into the column after each cycle, suggesting the possible abundance of active keratinase in the crude extract. The fractions eluted with the elution buffer (EF) herein referred to as the purified fraction were collected from each purification cycle and pooled. The EF fraction from the second purification cycle showed the highest specific activity and purification factor (Table 3.4) which suggests that the most active purified keratinase fraction was collected in this cycle. Therefore, EF fraction of the second cycle is most suitable for characterisation purposes.

The molecular weight of *Bacillus licheniformis* N22 keratinase was determined to be \approx 28 KDa by SDS-PAGE with 12 % gel (Fig. 3.13) and MALDI TOF-MS (Fig. 3.14). The single band and mass peak obtained by SDS-PAGE and MALDI TOF-MS respectively, indicates that the purified keratinase is homogeneous and monomeric (has no subunits). The obtained molecular weight of *Bacillus licheniformis* N22 keratinase is very close to that of *Bacillus pseudofirmus* FA30-10 (27.5 KDa; Kojima *et al*, 2006) but significantly different from that of *Bacillus licheniformis* PWD-1 (33 KDa; Lin *et al*, 1992). However, how the molecular weight of keratinases influences their functionality and activity, for instance in prion degradation, have not been described. It is interesting to note that proteinase K (28 KDa; Jany and Mayer, 1985), keratinase of *Bacillus licheniformis* PWD-1 and keratinase of *Bacillus licheniformis* N22 shown to degrade prion protein are within the molecular weight of the protease-resistant prion (27-30 KDa). It has been suggested that the chemical structure of keratinases with molecular weight <35 KDa may consist of only catalytic domain (Kim *et al*, 2004), hence may be more catalytically active.

Peptide mass fingerprint of trypsinised N22 keratinase (Fig. 3.15 A-C) was unmatched in Mascot search engine suggesting that this keratinase has not been previously characterised and documented. Therefore, the mass fingerprint provides useful reference information for comparison with other keratinases. Currently, there are no reports on the characterisation of keratinases by MALDI-TOF MS except for our earlier report on the determination of molecular weight of N22 keratinase (Okoroma *et al*, 2008) and recently the determination of molecular weight of *Bacillus* sp 50-3 keratinase by Zhang *et al* (2009).

Bacillus licheniformis N22 keratinase was stable at 4 °C for up to 8 weeks (section 3.1.8) compared to *Bacillus licheniformis* PWD-1 keratinase reported to loss 22 % of its activity after 19 days when stored at 4 °C (Shih and William, 1992). Hence, *Bacillus licheniformis* N22 keratinase has a considerably longer shelf life. Keratinase of *Streptomyces fradiae* has also been reported to retain its activity for several weeks at 4 °C when stored at pH 7 but rapidly lost its activity at pH 8.5 (Nickerson and Noval, 1961). Therefore, temperature and pH are important physical factors that affect the activity and stability of keratinases. Typically, proteases of *Bacillus licheniformis* species are known to be highly stable and retain their activity in environments containing potentially inhibitory chemical agents (Veith *et al*, 2004), and are therefore quite robust for various applications.

The chemical agents such as EDTA and Triton X-100 were found to enhance N22 keratinase activity (Table 3.3) similar to observed effect on keratinase of *Streptomyces* BA₇ (Korkmaz *et al*, 2003; Tapia and Simoes, 2008). The biochemical interaction of

different keratinases with chemical agents varies, and is used to classify keratinases (Giongo *et al*, 2008; Tatineni *et al*, 2008). For example, while EDTA enhances keratinase N22, it has been reported to partially inhibit keratinases of *Bacillus licheniformis* HK-1 (Rozs *et al*, 2001) and *Streptomyces* sp. S.K₁₋₀₂ (Letourneau *et al*, 1998). Interestingly, EDTA has been reported to enhance keratinolytic activity and at the same time inhibit proteolytic activity (Chao *et al*, 2007), suggesting that keratinolysis and proteolysis are different process and may indeed occur via different degradation pathways. The most significant enhancing effect on N22 keratinase activity was measured with DTT. On the contrary, SDS slightly inhibited N22 keratinase activity similar to some other keratinases (Korkmaz *et al*, 2003; Riffel *et al*, 2007; Tatineni *et al*, 2008; Tapia and Simoes, 2008). This suggests that SDS or similar chemical surfactants/detergents may inhibit keratinase activity in enzyme-surfactant/detergent formulations used for prion degradation, and hence it is necessary to determine their specific effects in such compositions.

4.2.6 Biosurfactant as a suitable biological detergent

The role of chemical surfactants/detergents in enhancing enzymatic prion degradation efficiency has been widely reported (Cho, 1983; Langeveld *et al*, 2003; Jackson *et al*, 2005; Suzuki *et al*, 2006; Croud *et al*, 2008; Pilon *et al*, 2009). However, it has been suggested that for the enzymatic method of prion degradation to be effective and economically viable for potential large scale application, low cost biological (natural) alternative to chemical detergents are desirable, for instance, in the decontamination and/or derivation of animal protein from animal by-products (Langeveld *et al*, 2003). This important knowledge gap has been filled by the use of biosurfactants as natural additive in enzymatic degradation of prion herein reported. Biosurfactants are particularly useful and advantageous because of their environmentally friendly properties (e.g. low toxicity, high biodegradability) and their biochemical properties enabling lowering of surface tension and unfolding of prion structure, and hence subsequent keratinase access and prion scission. In addition, the property of biosurfactants in inhibiting pathogen adhesion and formation of biofilms on steel surfaces (Muthusamy *et al*, 2008; Brzozowski *et al*, 2011), therefore provides a double advantage for their use in decontamination of prion contaminated steel surfaces such as medical devices. The use of biological detergents also prevents undesirable chemical load in enzymatic degradation processes, especially in large scale applications, and hence forestall chemical effluent entering the environment, or the need for additional facilities for effluent treatment.

The major limitations of use of biosurfactants in large scale applications include the low levels of production, and the high recovery and purification costs associated with

derivation of pure biosurfactants. *Pseudomonas aeruginosa* NCIMB 8626 crude biosurfactant was used in this study, providing substantial savings on cost and labour associated with purification regimes. This crude biosurfactants was stable and remained active for more than a year (section 3.1.9), which is beneficial in terms of laboratory time and cost savings in preparing fresh stocks. Crude biosurfactants may basically consist of protein, polysaccharide and phosphate in different ratio (Brzozowski *et al*, 2011). The protein and rhamnose (polysaccharide) concentrations of this crude biosurfactant preparation measured as BSA and rhamnose equivalents respectively (section 3.1.9). In a previous study, this biosurfactant was used to enhance the biodegradation of polycyclic aromatic hydrocarbon (Okoroma, 2006).

4.3 Degradation of keratin substrates and scrapie prions by keratinase

4.3.1 Degradation of keratin substrates

Keratinases are able to disassemble the tightly packed structure of β -keratin, exposing cleavage sites to proteolytic attack and hydrolysis (Kim *et al*, 2004). *Bacillus licheniformis* N22 keratinase extensively degraded keratin azure substrate, releasing substantial amount of azure dye into the reaction solution (Fig. 3.17), suggesting that this keratinase is potentially able to degrade other highly stable keratineous substrate such as feather keratin and hair. To further investigate this, melanised feather was digested with N22 keratinase in a feather degradation experiment.

Melanised feather was cleaned by washing with sterile distilled water in order to retain the keratin structure in its native state (Cortezi *et al*, 2008). Thus, the feathers used remained structurally uncompromised and free from microbial contamination (data not shown) which may aid degradation. Sterilisation by autoclaving denatures and degrades feather keratin making them lose their insolubility and resistance, hence become more susceptible to proteolytic attack (Suzuki *et al*, 2006; White *et al*, 1950; Gunderson *et al*, 2008). The feather substrates used in the feather degradation experiments were melanised (coloured) feathers. Melanised feathers are generally more resistant to enzymatic attack than white feathers as a result of the pigment, melanin, which has been reported to bind and inactivate keratinases and help to protect feather degrading bacteria (Goldstein *et al*, 2004; Gunderson *et al*, 2008) and other proteases (Suh and Lee, 2001; Kuo and Alexander, 1967). In addition, distal end (containing mainly barbs and barbules) of relatively mature melanised feather substrates were used instead of the proximal region where the rachis and calamus (see physical structure of feather: appendix 3) make up most of the mass (Gunderson *et al*, 2008). There are hardly any reports on the evaluation of keratinolytic activity using melanised feather substrate, and in most reported feather degradation

experiments, the feather substrate used were usually young and downy white feathers (Korkmaz *et al*, 2003; Bockle *et al*, 1995; Brandelli and Riffel, 2005; Cao *et al*, 2008; Zhang *et al*, 2009).

The cell-free crude keratinase extract of *Bacillus licheniformis* N22 fully degraded melanised feather (Fig.3.18). The residual feather (mainly rachises) was nonetheless soft and brittle and therefore handled with care during drying and weighing. The rachis constitutes most of the total feather mass and has been reported to be resist degradation by *Bacillus licheniformis* (Ramnani *et al*, 2005). The feather in the negative control experiment (incubated in distilled water) retained its weight after incubation suggesting that the feather was not degraded or dissolved in the distilled water. However, the feather in the control experiment containing denatured crude keratinase weighed slightly less than the starting mass after incubation suggesting that the feather may have been digested by residual keratinase activity. Interestingly, both diluted and undiluted crude keratinase were equally effective in degrading native melanised feather, suggesting that this keratinase is highly efficacious (Fig 3.18 A-B).

Feather degradation by keratinases has been mostly performed in the presence of suitable reducing agents such as live cells or chemical reductants (Brandelli and Riffel, 2005; Ramnani and Gupta, 2007; Liang *et al*, 2010). Cell-free keratinases of *Streptomyces* BA₇ (Korkmaz *et al*, 2003), *Streptomyces* S7 (Tatineni *et al*, 2008) and *Bacillus licheniformis* ER-15 (Tiwary and Gupta, 2010) have been reported to degrade feather in 24 h, 97 h and 12 h respectively. However, only about 10-20 % degradation is reportedly achieved by cell-free keratinases in the absence of reductants (Hossain *et al*, 2007). In addition, most purified keratinases are unable to effectively degrade native keratin for reasons which include: (1) the high degree of disulfide bonds in the keratin molecules (Bockle *et al*, 1995; Bressollier *et al*, 1999; Ramnani *et al*, 2005; Gupta and Ramnani, 2005; Riffel *et al*, 2007), and (2) removal, during purification, of fermentation culture constituents capable of reducing or breaking disulphide bonds (Cao *et al*, 2008). The later suggests that the presence of a consortium of enzymes in crude fermentation broth may be required to enhance feather keratin degradation (Sharma and Gupta, 2010). However, purified keratinase of *Bacillus licheniformis* N22 was able to fully degrade melanised feather in the absence of reducing agents, suggesting that this keratinase does not require reducing agents in order to effectively degrade highly stable keratin structure.

Considering that feather keratin and prions are structurally similar in terms of their β -sheet composition (Shih, 2002; Langeveld *et al*, 2003), degradation of melanised feather by N22

keratinase indicated its potential for prion degradation. Although the mechanism of enzymatic degradation of β -sheets of silk keratin and β -amyloids of prion have been reported to be significantly different (Numata *et al*, 2010), feather keratin as a model substrate for studying keratin degradation mechanisms and for identifying potential prion-degrading microorganisms remains highly relevant.

The extremely large amount of feather waste produced by the poultry industry poses serious disposal and pollution problems. In the United States, around 4 billion pounds of feather waste is generated annually (Parkinson, 1998; Schmidt, 1998) and the United Kingdom generates about 330 million pounds of feather waste (United Kingdom Food and Drink Processing Mass Balance (2004)). Feather is highly stable and has a melting point of around 230-240 °C (Schmidt, and Line, 1996). The disposal of feather waste by burning is a tough and an environmentally polluting process, and land filling requires a lot of land space which is not economically viable and environmentally sustainable as feathers decompose very slowly in the environment. However, feather wastes are potentially valuable and readily available raw material for the production of livestock feed supplements and amino acids (Onifade *et al*, 1998; Odetallah *et al*, 2003). Current feather waste processing methods such as steam pressure, chemical treatment and feather milling are cost and labour intensive, diminish product nutritional value and destroy important amino acids. For example, chicken feather conversion to feed supplement by cooking and sterilisation at high temperature and pressure followed by drying and grounding into powdered feather meal reduces the overall protein quality in addition to being a time consuming process (Wang and Parson, 1997; Shih, 1993; Dalev *et al*, 1997). Similarly, the current dehairing process in leather processing involves the use of extremely toxic sodium sulphide which also emits obnoxious odour as well as being expensive. Indeed, an estimated \$1 million is spent daily for treatment of tanneries waste worldwide (Macedo *et al*, 2005).

In recent times, attention has been drawn to microbial/enzymatic degradation as a desirable and valuable alternative for keratin waste processing (Yamamura *et al*, 2002; Korkmaz *et al*, 2003; Brandelli and Riffel, 2005; Gunderson *et al*, 2008). Enzymatic feather degradation is particularly important because it is simpler to operate than bacterial fermentation that requires maintaining the cell growth conditions (Ramnani and Gupta, 2007). The use of enzymes for dehairing is not destructive and does not modify the dermis hence results in high-value leather. Furthermore, low levels of organic matter results in the wastewater, thus reducing effluent treatment cost and risks of environmental pollution. The ability of *Bacillus licheniformis* N22 keratinase to degrade feather keratin therefore

suggests its potential for use in the conversion of feather waste to useful bio-products, as well as in the processing of other keratinized wastes.

4.3.2 *In vitro degradation of prion protein*

4.3.2.1 *Western Blot analysis and optimisation*

Prion protein in cell and tissue samples is commonly detected by Western blot using suitable anti-prion antibodies and characterised according to PrP-immunoreactive signal, PrP band pattern and size shift in molecular weight. The loss of or significant reduction in the PrP signal normally suggests a prion degradation event. PrP^{Sc} usually associated with prion infectivity is immunodetected after digesting prion sample with proteinase K, which readily digests PrP^C.

The PrP^{Sc} intensity on Western blot may depend on the the concentration and amount of sample assayed, the type of antibody used for detection, the procedures adopted and materials used. Optimisation of Western blot enabled determination of procedural conditions and suitable materials such as membrane blocking buffer and membrane blocking duration, which prevented cross-reactions, non-specific binding and unnecessarily long blocking duration, and improves sensitivity. *Bacillus licheniformis* N22 keratinase-bond Polyvinylidene Fluoride (PVDF) membrane blocked with BSA cross-reacted with the secondary antibody (anti-mouse IgG horseradish peroxidase from sheep) (Fig. 3.19). Interestingly, this cross-immunoreaction was eliminated when skim milk was used as the blocking agent. The reason for such cross-reaction has not been identified but may be related to epitopes binding (Kim, 1993). Because this reaction is specific to BSA, bio-conjugation of certain constituent of BSA with keratinase may have resulted in the observed cross reaction. Furthermore, the membrane blocking step in WB is usually considered an important step for preventing non-specific binding. However, skipping this step did not seem to diminish the quality of the blots obtained but rather shortened the overall experiment time by 1 h. It is possible that sufficient membrane blocking may have been achieved with the 1 % skim milk solution in which the primary antibody was diluted.

PrP^{Sc} signal intensity on Western Blot profile is related to its abundance in a prion sample. Therefore, undetectable PrP^{Sc} signal generally suggest the absence, or where enzyme-digested, the complete degradation of PrP^{Sc}. However, it is not uncommon for PrP^{Sc} intensity to increase following partial proteolysis, as a result of increased epitopes exposure due to protein unfolding and partial PrP^{Sc} disintegration (Brun *et al*, 2004; Jackson *et al*, 2005; Thackray *et al*, 2007; Ramos-Vara *et al*, 2008 and Karapetyan *et al*, 2009). Similarly, increased PrP^{Sc} intensity was observed in this study when prion sample

was digested with low PK and keratinase concentrations, and at short digestion times. However, increasing the enzyme and digestion time resulted in higher degradation activity and loss of PrP^{Sc} intensity. Therefore, it can be argued that the increased PrP^{Sc} intensity captured the early stages of degradation activity (Fig. 3.23 and 3.24: Lane 2).

Monoclonal antibodies (mAbs) detect prion protein at different affinity levels and sensitivities by targeting and binding specific linear epitopes, and therefore recognise prion proteins from different species differently (Laffont-Proust *et al*, 2006). SAF83 mAb for instance recognises solid-phase immobilised peptide 126-164 (in the central region) but does not bind peptide 142-160 (Féraudet *et al*, 2005). SAF83 has high affinity for PrP to which it rapidly binds within 10 min as tested for recombinant human PrP (rhPrP) (Axela biosensor). High affinity to PrP results in high immunoreactive intensity, and hence shorter primary antibody incubation time (Ramos-Vara *et al*, 2008). SAF83 has been shown to be unable to recognise PrP in microcebus, marmoset, macaque and baboon, although it detects PrP in species used as animal models for study of prion diseases such as hamster, rat and mouse (Laffont-Proust *et al*, 2006). Therefore, SAF83 was adequate for detecting ME7 scrapie prion.

In addition, different monoclonal antibodies (mAbs) were evaluated for immunodetection of digested ME7 scrapie PrP^{Sc} in order to identify one of high affinity, and to ensure that no possible epitopes of PrP^{Sc} were missed (Fig. 3.28). The mAbs SAF83 and 9A2 demonstrated outstanding immunoreactivity with PrP^{Sc} compared to mAbs 12B2 and 6C2. The electrophoretic profiles of ME7 detected with mAbs SAF83 and 9A2 were similar, although the concentration of 9A2 used was 2.5 times higher than the concentration of SAF83 (section 2.3.3.2.3). The mAb SAF83 was selected for this study because of its high affinity and because it probes the central region of PrP.

4.3.2.2 *Degradation of ME7 scrapie prion*

The crude keratinase of *Bacillus licheniformis* N22 and the different fractions of the purification steps degraded scrapie prion differently; increasing in efficacy from the crude keratinase extract (S) through to the purified fraction (EF) (Fig. 3.21), indicating greater prion degrading activity with purity. While the purified keratinase (EF) was able to effectively digest ME7 scrapie prion at 50 °C in 2 h (Fig. 3.21), a combined purified keratinase (EF) and biosurfactant (BS) composition effectively degraded ME7 scrapie prion in a shortened digestion time of 10 min at 65 °C to levels undetectable by western blot analysis (Fig. 3.26), indicating the effects of the interplay of digestion time, temperature and biosurfactant in enzymatic prion degradation. EF alone was unable to fully digest ME7 prion at 65 °C in 1 h compared to complete digestion with EF+BS (Fig.

3.24), suggesting the significant effect of BS in the degradation mechanism. Furthermore, EF+ BS partially degraded ME7 prion at 50 °C in 1 h (Fig. 3.25) but completely degraded it to undetectable levels of PrP^{Sc} in 10 min by increasing the digestion temperature to 65 °C (Fig. 3.26), clearly indicating the significant thermal effect on ME7 prion degradation. However, ME7 scrapie has been reported to be thermally stable up to 84 °C (Somerville *et al*, 2002). Whether digestion at 65 °C destabilised ME7 scrapie material or increased keratinase activity or both, temperature appears to be a critical factor in the interactions of the EF-BS-ME7 reaction complex. The catalysis of keratinases under thermophilic conditions and solubilisation of prions by biosurfactant certainly enhanced the degradation process. The degradation mechanism for the EF-BS-ME7 complex probably includes unfolding the PrP^{Sc} structure, weakening the structural bonds, facilitating PrP^{Sc} solubilisation and allowing increased proteolytic access and PrP^{Sc} susceptibility to keratinase activity (Langeveld *et al*, 2003; Bolton *et al*, 1984; Oesch *et al*, 1994). Specifically, biosurfactant may be involved in lowering surface tension of the reaction mixture, solubilising the prion substrate and increasing its surface area, thereby allowing keratinase greater access in cleaving the prion substrate. Therefore, the hydrophobic moiety of the biosurfactant may be attach to the prion molecules and pulled by the hydrophilic head causing disruption of intact PrP^{Sc} aggregates and stretching it out to allow easier scission of the stabilising disulphide bonds by keratinase. The disulphide bond normally stabilises the folded form of protein by forming a nucleus of hydrophobic core. Although proteases such as PK cleave proteins non-specifically, they show preference to cleave hydrophobic and aromatic amino acids (Kraus *et al*, 1976).

Crude biosurfactant (BS) alone was unable to degrade ME7 scrapie prion as detected by Western blot analysis (Fig. 3.24), suggesting that it lacked proteolytic activity and prion degrading potential. Although some *Pseudomonas* strains produce keratinases (Sharma and Gupta, 2010; Tork *et al*, 2010), and a *Bacillus licheniformis* strain have been reported to concomitantly produce alkaline protease and biosurfactant (Ramnani *et al*, 2005), the crude biosurfactant of *Pseudomonas aeruginosa* NCIMB 8626 exhibited no significant keratinase activity and keratinase of *Bacillus licheniformis* N22 lacked biosurfactant properties. Therefore, the EF and BS appeared to play different but specific roles which resulted in an interestingly remarkable synergistic enzymatic degradation of ME7 prion.

In the time-course experiment, the loss of the glycosylation band (Fig. 3.25) suggests the extent of cleavage of PrP^{Sc} by the enzymatic preparation (EF+BS) and illustrates the interplay of incubation time and temperature in the prion degradation process. The decision to choose a shorter incubation time over higher temperature or vice versa for enzymatic

prion degradation will depend on the type of application (e.g. decontamination of sensitive surgical device and remediation of prion contaminated soil) and on economic considerations.

Significant level of PrP^{Sc} degradation at low concentrations of keratinase (EF) (up to 1:50 dilutions) (Fig. 3.27), and the effective degradation of feather at dilute crude keratinase extract (1:5 dilutions) (Fig. 3.18A) suggests the efficacy of this keratinase. This is also important in economic terms as much less material is required to achieve similar levels of result such that using higher concentrations is a waste of resources. It has been reported that higher protease concentration does not necessarily increase the rate of PrP^{Sc} degradation (Lawson *et al*, 2007; Yoshioka *et al*, 2007),

The enzymatic degradation at mild digestion conditions is of general interest in the decontamination of sensitive medical devices, animal products [Meat and Bone Meal (MBM) and Specific risk materials (SRM)] and the prion contaminated environment. The inability to achieve efficient prion degradation at mild digestion conditions (neutral pH, moderate temperature, and low enzyme concentration), currently limits the use of enzymatic decontamination methods in economic and operational terms (Pilon *et al*, 2009). The enzymatic degradation method described here, degraded ME7 scrapie prion under moderate physical condition (pH 7 and 65 °C) and short digestion time (10 min) (Fig. 3.26). In addition, it did not require a truncated multi-step approach (e.g. Langeveld *et al*, 2003; Jackson *et al*, 2005; Croud *et al*, 2008) in which the infectious material is either pre-treated and/or digested with multiple enzymes or in the presence of chemical surfactants or alkali carrier such as NaOH. This enzymatic method is efficient and practical, and the constituent agents (N22 keratinase and biosurfactant) are purely biological agents of potentially low production cost. Therefore this method could provide a good, environmentally acceptable, economically sound and safe alternative to existing prion decontamination methods.

4.4 Residual infectivity evaluation of keratinase-digested scrapie prions

4.4.1 Scrapie cell assay

Scrapie cell-based scrapie assay has become an increasingly important and reliable method for evaluating residual infectivity, characterising prion infectivity, prion strain typing and infectious titre estimation (Klohn *et al*, 2003; Mahal *et al*, 2007; Edgeworth *et al*, 2009; Neale *et al*, 2010; Edgeworth *et al*, 2011). Cell-based assay is particularly attractive for its advantages of high sensitivity, short assay time (about three weeks) compared to mouse bioassay, and reduced labour and cost requirement. While Western blot analysis is the

most common biochemical method for prion immunodetection, the presence of prion infectivity in the apparent absence of detectable levels of PrP^{Sc} (Berardi *et al*, 2006; Barron *et al*, 2007; Lasmezas *et al*, 1997) limits its use for evaluating residual prion infectivity. Therefore, more consistent and sensitive methods such as scrapie cell assay and bioassay are necessary to validate results from Western blot analysis and to determine the efficacy of prion degradation methods.

The Western blot analysis of ME7 scrapie digested with the enzymatic treatment suggests the complete degradation of PrP^{Sc} (Fig. 3.27). To establish that the absence of PrP^{Sc} was accompanied by complete loss of infectivity, enzyme-digested samples were evaluated by scrapie cell assay using Rov9 cell line. The Rov9 cell line is susceptible to a number of scrapie strains including SSBP/1 (Neale *et al*, 2010). Since cell lines susceptible to ME7 prion strain were not available for this study, SSBP/1 scrapie infectivity was evaluated by standard scrapie cell assay (SSCA) using Rov9 cells. Both ME7 and SSBP/1 scrapie strains were completely digested by EF+BS enzymatic treatment to levels of PrP^{Sc} undetectable by Western blot (appendix 4), suggesting similar levels of prion degrading activity. Therefore, the results of enzymatic digestion of SSBP/1 and ME7 were considered to be comparable.

The result of the scrapie cell assay demonstrates that the enzymatic treatment effectively destroyed prion infectivity as shown by the inability of the enzyme digested SSBP/1 brain homogenate to infect Rov9 cells (Fig. 3.29 and 3.30). Keratinase alone effectively destroyed SSBP/1 infectivity similar to the total loss of PrP^{Sc} signal intensity in Western blot profile following extended digestion of ME7 scrapie. Digestion of SSBP/1 with BS resulted in a significant reduction in the number of infected cells which suggests that SSBP/1 infectivity was significantly attenuated but not enough to prevent infection of cells. This is particularly interesting as Western blot analysis of BS-digested ME7 scrapie did not indicate detectable loss of PrP signal intensity relative to undigested control. This may be explained by a higher sensitivity of the scrapie cell assay. In addition, SSBP/1 scrapie prion infectivity was significantly attenuated at the incubation temperature (65 °C) of the enzymatic digestion, but not significantly to completely destroy prion infectivity (Fig. 3.30). The number of spot counts detected in the EF and EF+BS treatment groups (Fig. 3.30) were probably background noise as confirmed by absence of visually detectable infected cells on the photographic image of the ELISPOT plate (Fig. 3.29).

The inability of the enzyme-digested scrapie substrate to propagate infectivity in the susceptible Rov9 cells suggests the complete loss of SSBP/1 infectivity. Therefore, the

efficacy of this enzymatic treatment method for complete destruction of prion infectivity has been clearly and reliably validated by the scrapie cell assay. This result suggests that the loss of detectable levels of PrP^{Sc} signal in the enzyme-digested scrapie prion as determined by Western blot correlated with loss of prion infectivity as determined by the scrapie cell assay. Therefore, the scrapie cell assay is a potential method for evaluating residual prion infectivity.

4.4.2 Bioassay

The level of PrP^{Sc} present in prion sample homogenates is determinable by Western blot analysis and, cell culture assay is a reliable method for evaluating residual prion infectivity. Currently, rodent bioassay is considered the gold standard for the evaluating residual prion infectivity. Therefore, to further investigate the efficacy of the enzymatic treatment method described here for destroying prion infectivity, enzyme-digested ME7 scrapie was evaluated for residual infectivity by mouse bioassay using transgenic (Tga20) mice.

The incubation time of ME7 scrapie prion inoculated intraperitoneally has been reported to be approximately 94 days in transgenic mice (Karapetyan *et al*, 2009), and approximately 274 ± 5 days in wild-type (C57BL) mice (Brown *et al*, 2009). The short incubation time of the disease in transgenic mice is attributed to high levels of endogenous PrP^C expression (about 8 fold more PrP^C in heterozygous Tga20/wild type and 16-fold higher for those with diploid dose of Tga20). PrP^C is a substrate for prion conversion and abundant levels of PrP^C in transgenic mice results in higher rate of PrP^{Sc} conversion and prion propagation, and hence shortened incubation time of disease (Fischer *et al*, 1996).

In the mouse bioassay, the long incubation times observed was characteristic of wild type rather than transgenic mice. Western blot profiles showed that the PrP^C intensity of the Tga20 were variable, and were not significantly higher than those of the wild type mice (Fig. 3.34). While the Western Blot may also suggest slight differences between loading of PrP and β -actin (loading control), which was less likely, the slight differences may simply reflect more or less areas of the brain stem with low PrP and actin high axon fibre tracts hence the PrP in the Tga20 cannot reflect 8 or 16 times higher PrP such that the test mice is C57BL/6 (Prof. Roger Morris-Personal Communication). It is possible that the Tga20 mice may have lost their transgene during outbreeding to C57BL/6 mice over several generations, resulting in characteristically wild-type mice. Therefore, the loss of transgene, genetic shifts and variable levels of PrP^C expression in the experimental mice may have

significant effects on their susceptibility to prion infection and propagation of disease as this experiment has shown.

To allow for direct comparison of the survival time data in the bioassay, only mice that were culled/died at ≤ 550 days, and those that were PrP^{Sc} positive as determined by Western blot analysis of brain homogenates were included in the survival time analysis. The incubation time in IBH Neat group was found to be 278 ± 9 days which is in close agreement with 274 ± 5 days reported by Brown *et al* (2009) for ME7 inoculated intraperitoneally in C56BL (wild-type) mice.

Two clearly distinct sub-populations of mice with highly significant difference in survival time occurred in the IBH digested treatment group. One sub-population (47 % of all the mice inoculated) had a mean survival time of 208 ± 27 days. Such shortened incubation time would suggest rapid progression of disease incubation with accompanying PrP^{Sc} accumulation. However, the mice in this sub-population were all PrP^{Sc} negative suggesting that they were either infected or may be harbouring undetectable trace levels of infectivity. Disease incidence < 100 % is considered to suggest presence of undetectable or trace levels of infectivity (Brown *et al*, 2009), and IBH digested group had a disease incidence of 53 %, suggesting possible undetectable trace levels of infectivity. The mean survival time in this sub-population was significantly lower than that of IBH Neat group suggesting that they are likely to have died of other causes or the mice were mistakenly culled too early (Fryer and McLean, 2011), which further justifies their exclusion in the survival time analysis. Significant increase occurred in the survival time for the IBH digested group (PrP^{Sc} positive sub-population) compared to the IBH Neat (positive control) group.

Put together, it can be concluded that enzymatic digestion of ME7 substantially destroyed infectivity as lack of detectable levels of PrP^{Sc} in the brain of challenged mice in the IBH digested group would suggest, or ME7 prion infectivity was considerably reduced following enzymatic digestion resulting in significantly increased survival time in challenged mice from 278 ± 9 days to 334 ± 42 days. Therefore, EF+BS has not only degraded PrP^{Sc} in the form seen on Western Blot, but also substantially destroyed or reduced the infectious titre of digested ME7 prion.

4.5 Potential applications of this enzymatic treatment method

Keratinase of *Bacillus licheniformis* N22, especially in a composition with biosurfactant of *Pseudomonas aeruginosa* showed a remarkable efficacy for prion degradation at mild temperatures and short incubation. The potential applications of this enzymatic method

may include the decontamination of delicate and reusable surgical and dentistry instruments, processing of SRM and MBM, composting of animal carcasses and animal wastes as fertilisers, disposal of sheep scrapie, decontamination of infected materials and all forms of clinical wastes prior to disposal, remediation of prion contaminated environment (soil, water and wastewater), degradation of feather waste into poultry feed, organic fertilisers and amino acid production, and dehairing of hides in the leather industry.

A recent report comparing the efficacy of three commercially available prion decontamination reagents (HAMO 100 [HPID Steris]; Rely⁺ On PI [DuPont Corporation] and Prionzyme [Genencor]) demonstrated high variability in their effectiveness (Edgeworth *et al*, 2011). Rely⁺ On PI and Prionzyme decontaminated steel-bound scrapie to levels of infectivity undetectable by standard steel-binding assay (SSBA) at 50 °C and 65 °C respectively. Rely⁺ On PI is composed of a mixture of three different undisclosed packs of substances and Prionzyme was composed of 2M NaOH (commonly reported chemical method), which on its own was able to decontaminate steel-bound scrapie. This report suggests that an effective and truly biological (enzymatic) treatment method remains highly desirable. Therefore, the enzymatic treatment method reported in this research holds significant commercial prospect.

4.6 Conclusion

A standardisation protocol for commercial keratin azure has been reported which results in a highly homogeneous substrate, making it a reliable substrate for consistent and reproducible keratinase activity measurement useful for evaluation and comparison of keratinases. Also, two simple but useful assays (spot inoculation and the hydrolysis ring assays) have been introduced for the screening and characterisation of proteolytic and keratinolytic microorganisms and their proteases. Keratinase assay and a PK calibration curve baseline method established for selecting potential prion degrading microorganism proved to be successful.

A novel strain of keratinase producing *Bacillus licheniformis* has been isolated and characterised. This bacterial strain identified and designated as *Bacillus licheniformis* N22 has been deposited with National Collection of Industrial Food and Marine Bacteria (NCIMB) with ascension number NCIMB 41708. The keratinase of *Bacillus licheniformis* N22 completely degraded melanised feather and was able to degrade ME7 scrapie prion. N22 keratinase formed a remarkable synergistic activity with biosurfactant of *Pseudomonas aeruginosa* NCIMB 8626 and effectively degraded ME7 and SSBP/1 scrapie

strains under mild digestion conditions and short incubation time. The complete destruction of sheep scrapie infectivity is particularly important in terms of disposal of sheep scrapie. In a preliminary test, other field scrapie prions have also been successfully degraded by this enzymatic treatment (appendix 4). These results suggest that this enzymatic treatment method has potential application in feather processing as well as prion decontamination, and could be integrated into existing medical devices sterilisation process.

4.7 Recommendations for future work

To further evaluate the efficacy of this enzymatic treatment method on ME7 scrapie and other scrapie strains, it is recommended to carry out bioassay using transgenic (Tga20) mice that has been genetically profiled and confirmed to produce high levels of PrP^C, and a statistically significant number of mice (at least 16 mice) is required per treatment group (Dr Oduola Abiola- Personal Communication). In addition, the robustness of this enzymatic treatment method on other types of field prion (BSE, CWD and vCJD) strains should be investigated.

It is also recommended to investigate the efficacy of this enzymatic method for treatment of prion contaminated steel material. This is particularly important for purposes of application in decontamination of medical devices and laboratory equipments. Furthermore, the remediation of prion contaminated soil is becoming increasingly important, hence it is recommended to investigate the potentials of this enzymatic treatment method for the treatment of prion contaminated soil for the purposes of application in the remediation of farms with the history of TSE occurrence and landfills that may have received prion contaminated carcasses, such as during the onset of BSE outbreak in the UK. In addition, other potential biotechnological applications of this enzymatic treatment method such as feather to feather meal conversion and hides processing for leather production could be explored.

Finally, it is recommended to carry out detailed structural mapping of keratinase N22 to gain in-depth knowledge of the biochemical nature of this enzyme, and also to investigate the mechanism of the interactions between keratinase, biosurfactant and prion in the enzymatic degradation reaction. In addition, analysis of the constituents of the enzymatic degradation product will help to identify at what points the enzyme cleaves specific prion strain and provide insight into the mechanism of the alteration of prion infectivity.

4.8 References

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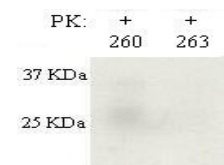
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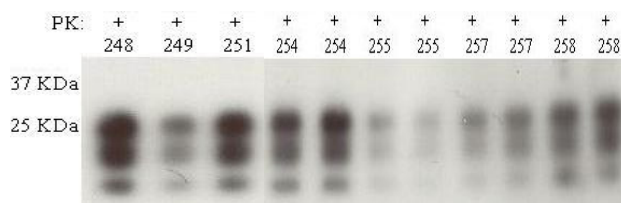
Appendices

Appendix 1: Western blot profiles of brain homogenates of culled mice from the different treatment groups of the mouse bioassay. **Key:** NBH (Normal brain homogenate); IBH (Infectious brain homogenate); PK (Proteinase K) and Number (Brain homogenate of mouse identified by that number). All immunodetection were carried out with SAF83. Appendix 1 is referred to in section 3.3.2 (mouse bioassay).

Brain homogenates of animals inoculated with NBH digested with enzymatic treatment



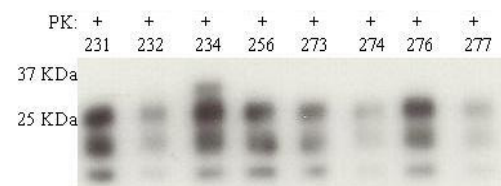
Brain homogenates of animals inoculated with neat IBH



Brain homogenates of animals inoculated with 1:10 dilution of IBH



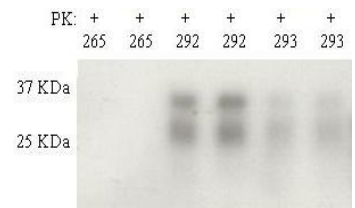
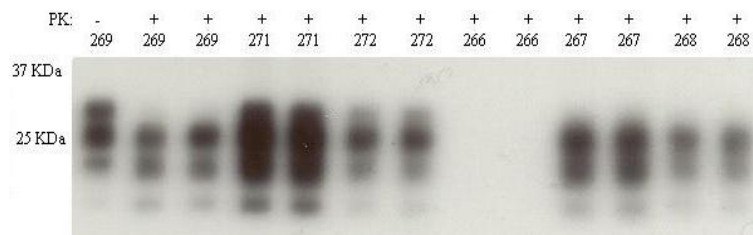
Brain homogenates of animals inoculated with 1:100 dilution of IBH



Brain homogenates of animals inoculated with 1:1000 dilution of IBH

ALL MICE ALIVE AT TERMINATION OF EXPERIMENT

Brain homogenates of animals inoculated with IBH digested with enzymatic treatment



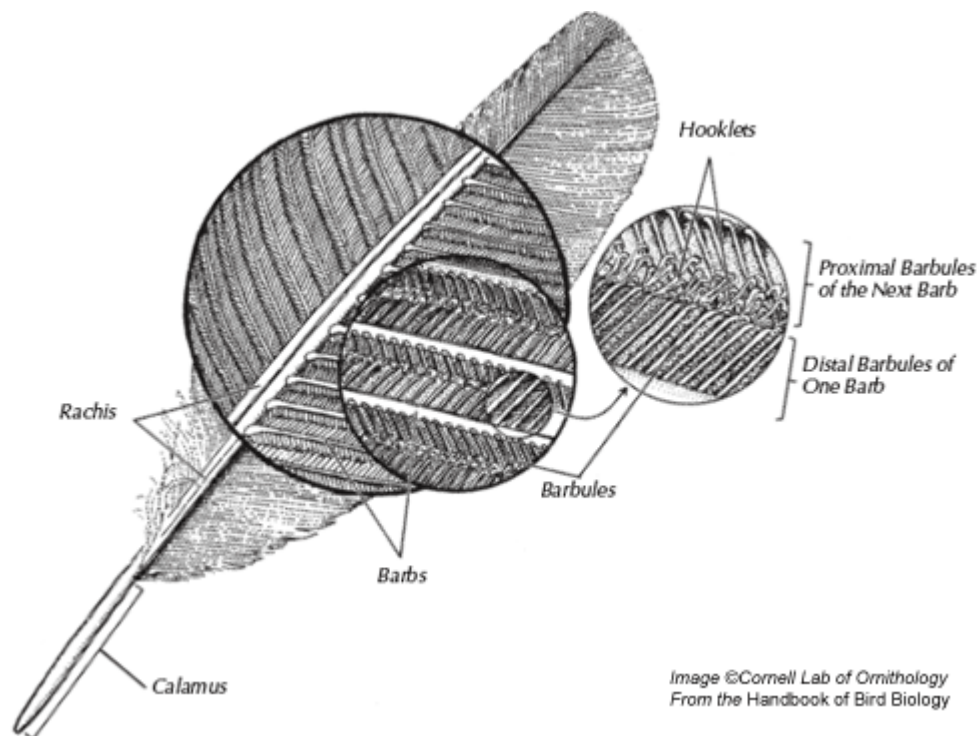
Appendix 2: Summary of bioassay mice indicating individual mouse identification, sex type, inoculation batches, survival time post-inoculation and PrP^{Sc} status of brain homogenates from culled mice. A total of 55 mice were inoculated out of which 37 mice were culled /died. All mice that survived beyond 550 days were not tested for PrP^{Sc}. Appendix 2 is referred to in section 3.3.2 (mouse bioassay).

Treatment groups	Identification no. and sex of mice inoculated	Survival time (days)	Detected PrP^{Sc} (-/+)
NBH (Digested) (-ve control)	260 M	384	-
	261 M	>550	Not tested
	262 M	>550	Not tested
	263 F	496	-
	264 F	>550	Not tested
IBH (Neat) (+ control)	248 F	296	+
	249 F	283	+
	251 F	276	+
	254 F	275	+
	255 F	275	+
	257 F	269	+
	258 F	275	+
IBH (1:10 dilution) (+ control)	287 F	204	+
	288 F	259	+
	289 F	303	+
	290 F	>550	Not tested
	295 F	>550	Not tested
	283 M	217	+
	284 M	321	+
	285 M	>550	Not tested
IBH (1:100 dilution) (+ control)	286 M	>550	Not tested
	231 M	343	+
	232 M	340	+
	233 M	>550	Not tested
	234 F	334	+
	235 F	>550	Not tested
	236 F	>550	Not tested
	256 F	345	+
	273 M	334	+
	274 M	336	+
	275 M	>550	Not tested
	276 F	328	+
	277 F	369	+
	278 F	>550	Not tested
IBH	244 M	>550	Not tested

(1:1000 dilution) (+ control)	245	M	>550	Not tested
	246	M	>550	Not tested
	247	M	>550	Not tested
	252	M	>550	Not tested
	253	M		Not tested
IBH (Digested) (Experimental Group)	269	F	325	+
	271	F	296	+
	272	F	299	+
	265	M	181	-
	266	M	186	-
	267	M	321	+
	268	M	332	+
	279	M	191	-
	280	M	243	-
	281	M	342	+
	282	M	431	+
	291	F	191	-
	292	F	233	-
	293	F	233	-
	294	F	327	+

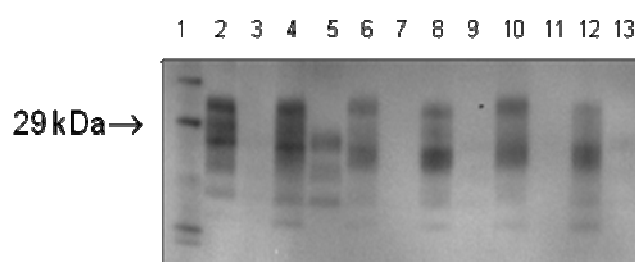
M- male; F- female; '+' PrP^{Sc} positive mice; '-' PrP^{Sc} negative mice or below detection limit

Appendix 3: The physical structure of feather which typically consists of a central shaft with a proximal end (calamus or quill) and a distal end (rachis). Attached to the rachis are barbs, to which barbules are attached. Tiny hooklets tie the barbules and consequently, the barbs together. Appendix 3 is referred to in section 4.3.1 (degradation of keratin substrate).



<http://www.birds.cornell.edu/AllAboutBirds/studying/feathers/feathers>

Appendix 4: Western Blot analysis of various various scrapie strain (1 % brain homogenate) samples digested with EF+BS and immunodetected with SHA31 mAb. Lanes 2, 4, 6, 8, 10 and 12 are undigested samples (Controls) and lanes 3, 5, 7, 9, 11 and 13 are the replicate corresponding samples digested with EF+BS. ME7 (lane 3) and SSBP/1 (lane 7) scrapie strains were completely degraded following digestion with EF+BS. Appendix 4 is referred to in section 4.4.1 (scrapie cell assay).



Lane	SampleID
1	Marker
2	ME7-
3	ME7+
4	PG 300/97-
5	PG 300/97+
6	SSBP1-
7	SSBP1+
8	PG 2413/98-
9	PG 2413/98+
10	PG 467/07-
11	PG 467/07+
12	PG 241/97-
13	PG 241/97+

Appendix 5: List of academic presentations

- **Okoroma, E.A.,** D. Purchase, H. Garelick, A. Jen, R. Morris and O. Abiola (2011). Degradation of infectious prion protein by a novel enzymatic treatment. School of Health and Social Sciences Annual Summer Conference. June 23, 2011.
- **Okoroma, E.A.,** D. Purchase, H. Garelick, R. Morris and O. Abiola (2011). Degradation of infectious prion protein by a novel enzymatic treatment. *Prion*. Vol. 5. Apr-Jun, 2011. P.108.
- **Okoroma, E A.,** Purchase D., Garelick H. and Abiola O. (2011). A novel enzymatic composition effective for prion degradation. Society for General Microbiology Spring Conference, Harrogate, UK. Apr. 11-14.
- **Okoroma, E.A.,** D. Purchase, H. Garelick, A. Jen, R. Morris and O. Abiola (2010). Novel bacterial keratinase for degradation of infectious prion protein. School of Health and Social Sciences Annual Summer Conference. June 24, 2010.
- **Okoroma, E.A.,** D. Purchase, H. Garelick, A. Jen, R. Morris and O. Abiola (2009). Degradation of scrapie infected brain homogenate by a novel bacterial keratinase. *Neuroprion Newsletter*, Prion2009 Special edition, 12-13.
- **Okoroma, E.A,** D. Purchase, H. Garelick, A. Jen, R. Morris and O. Abiola (2009). Degradation of scrapie infected brain homogenate by a novel bacterial keratinase. Prion 2009 International conference, Thessaloniki - Chalkidiki, Greece, Sept. 23-25, 2009.
- **Okoroma Emeka,** Diane Purchase, Hemda Garelick and Oduola Abiola (2009). Effect of temperature and pH on keratinase production in a novel feather degrading bacteria. 10th Symposium on Bacterial Genetics and Ecology, Uppsala, Sweden, June, 15-19, 2009.
- **Okoroma, E A.,** Purchase D., Garelick H. and Abiola O. (2009). Bacterial Keratinase: Prospects for prion degradation. Society for General Microbiology Spring Conference, Harrogate, UK, Mar. 30-Apr. 2.
- **Okoroma E A.,** Purchase D., Garelick H., Abiola A. and Abiola O. (2009). Keratinases and Prion inactivation: Possibilities from keratin degradation. Canada's Prion Conference, PrP^{CANADA} 2009, Edmonton, Alberta, Canada, Mar.1-3.
- **Okoroma E A.,** Purchase D., Garelick H. and Abiola O. (2008). Keratinase production by proteolytic microorganisms in activated sludge and farmyard waste. 163rd Society for General Microbiology Meeting, Ireland, Sept. 8-11.

- **Okoroma E.A.** (2007). Conference report: the 9th Symposium on Bacterial Genetics and Ecology, 23rd-27th June, 2007, Wernigerode, Germany. Journal of Health, Social and Environmental Issues Vol. 8, No 2, p 55-56.
- Purchase D., Garelick H., **Okoroma E.**, Christen H. and Abiola O. (2007). Keratin degradation by proteolytic microorganisms isolated from activated sludge and farmyard manure: possible application in decontamination of prion protein. 9th Symposium on Bacterial Genetics and Ecology, Wernigerode, Germany, June, 23-27.

In the adulthood, CRMP expression is maintained in brain areas retaining high plasticity [3]: as an example, CRMP1 is crucial for spatial learning and hippocampal plasticity [4]. Under pathological conditions, CRMPs have been proposed to play a role in neuronal death and neurodegenerative disorders: hyperphosphorylation of CRMP2 could be involved in the pathological aggregation of microtubule-associated proteins during Alzheimer disease [5, 6].

While recent studies suggest that synaptic alterations are first events in the mechanisms of prion-mediated neurodegeneration little is known on the identity of the neuronal plasticity-related genes potentially concerned. Thus in the present study we evaluated the possible implication of these CRMPs. The expression of 4 CRMPs was studied in the brain of C57Bl/6 mice infected with the BSE strain of prion agent. Using RT-PCR and Western-blot methods, CRMP-1, -2, -4 and -5 were analysed quantitatively in C57Bl/6 mouse brains at mid-course (90 days post inoculation (d.p.i.)) and at the terminal stage of the disease (180 d.p.i.) induced by BSE strain of agent injected by intra cerebral route.

At the terminal stage of the disease, gene expression of each CRMP had decreased, that most probably reflects a participation in a generalized response to severe damage within the brain. In contrast to the overall disturbance in CRMP expression, only CRMP-4 mRNA levels were significantly modified in the brainstem at the mid-stage of the disease. Interestingly, this increase was concomitant with higher levels of CRMP-4 proteins. This specific up-regulation of CRMP-4 expression pointed to clearer participation of this protein in prion pathogenesis since at that time the only neuropathological abnormalities detected was the limited PrP^{Sc} deposition. This over-expression may support the prion-initiated neurite disorganization or might promote the selective remove of damaged neuritis. Altogether our findings picked out originally CRMPs and especially CRMP-4 as credible actors in prion-induced neurodegenerative processes and already opens new insights into molecular mechanisms of prion pathogenesis.

This work was recently published in Brain research [7].

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Degradation of scrapie infected brain homogenate by a novel bacterial keratinase

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Emeka Okoroma

The resilient nature of infective prion has meant that they cannot be destroyed by common proteases or conventional sterilisation practices. Their affinity for and stability on metal surfaces has critical health implications for reusable surgical and dentistry instruments.



The poster prize winners

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Effective methods for prion decontamination have become increasingly desirable in order to manage the risks of prion infectivity and transmissibility. Incineration and alkaline hydrolysis are currently the most widely used means for destruction of the infective agent but these methods have obvious limitations of environmental acceptability, application compatibility, cost and loss of reusable materials. Most chemical and physical methods are harsh and inadequate for practical large scale application.

Enzymatic digestion of prions is considered as a mild, environmentally friendly, economically sound and safe method for the decontamination of prion infected materials, and was one of the science objectives and targets in UK Transmissible Spongiform Encephalopathy Directorate 2003-2006 Science Strategy [1]. Enzymatic degradation of prions provides a viable alternative for decontaminating animal carcasses, specified risk materials, medical instruments and laboratory equipments. A number of research effort on enzymatic degradation, inactivation and decontamination of infective prions have reported different levels of success under various experimental conditions; most of these methods required augmentation by preheating of prion contaminated tissue, addition of chemical surfactant/detergent and oxidizing agents, high alkaline pH, high temperature and long digestion time [2-6]. A stand-alone enzymatic alternative for prion degradation that is environmentally safe, compatible for use on sensitive materials, and suitable for practical use is highly desirable.

Our approach was based on the hypothesis that the structural similarity of feather keratin and infective prion in terms of their β -sheet proteic content may enable feather degrading bacteria to degrade prions. Proteolytic and keratinolytic microorganisms were isolated from farmyard waste and sewage sludge on feather meal agar. Of thirty-two isolates, one was selected on the basis of casein-agar hydrolysis assay, keratinase assay and Proteinase K equivalent curve for its ability to produce keratinase. The isolate was identified and designated as *Bacillus licheniformis* N22. Keratinase of this bacterium was purified by a step-wise feedback approach using the HiTrap Blue column (GE Healthcare), and the molecular weight determined as ≈ 28 KDa by SDS-PAGE. This novel keratinase demonstrated significant activity on keratin azure (11 U/mL) and

completely degraded melanised feather within 48h at 50°C (Fig. 1). Scrapie infected mouse (ME7) brain homogenate was digested with this keratinase at 50°C for 2h in the absence of chemical surfactant/detergents and without preheating the prion sample. PrP^{Sc} signal of the digested sample was reduced to a level undetectable by western blotting analysis (Fig. 2). Further optimisation of incubation conditions is currently ongoing, and future work includes degradation of BSE prions and evaluation of residual infectivity by animal bioassay.

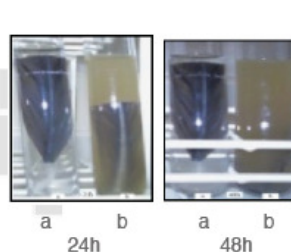


Fig. 1: Degradation of melanised feather
(a) Control (b) with keratinase

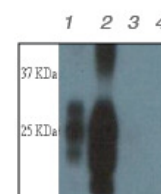


Fig. 2: Scrapie prion degradation analysed by western blotting.
Lanes:
1- Control; 2- with Proteinase K (100 μ g/ml);
3&4- with keratinase

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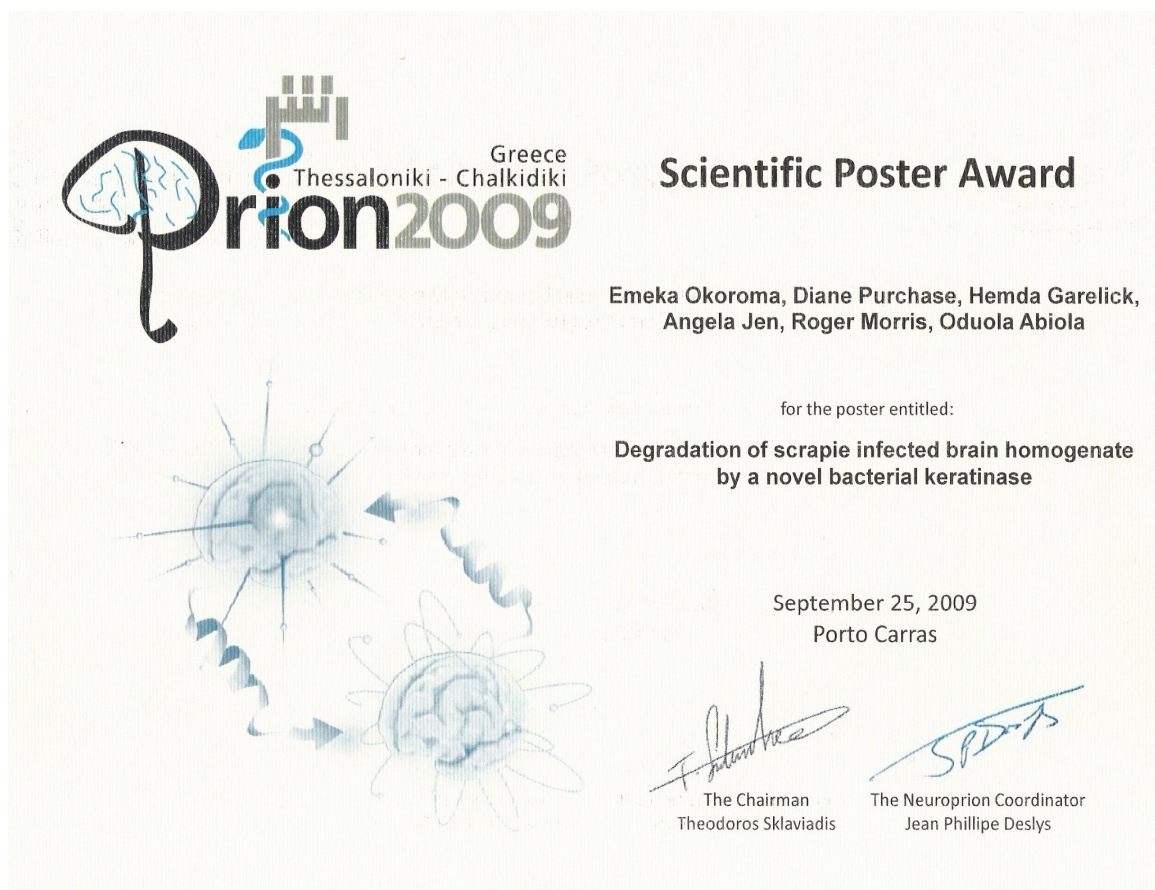


The poster prize winners

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Appendix 7: Scientific Poster Award for poster presented at the Prion2009 Congress, Thessaloniki, Greece.



Identification of a *Bacillus licheniformis* strain with profound keratinase activity for efficient feather waste management

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Abstract

Background: Significant amount of keratins in the form of feather, hair, hoof and horn are generated annually by the livestock industry. Keratinases are increasingly important in the reprocessing and environmental pollution control of keratin wastes. The aim of this study is to isolate a microbial strain of high keratinase activity and to evaluate its feather degrading potential.

Results: Thirty-two keratin degrading microbial strains from farmyard wastes and primary effluent were isolated using a selective medium containing feather meal at 30, 37 and 50 °C. The proteolytic activities of the isolates were determined using a casein medium and their abilities to degrade keratin were evaluated by the keratinase assay using keratin azure as the substrate. One of the isolates, which demonstrated the highest keratinolytic activity ($11.00 \pm 0.71 \text{ U mL}^{-1}$) was identified as a species of *Bacillus licheniformis* based on the 16S rDNA analysis and designated *Bacillus licheniformis* N22. Its keratinase activity was 58% higher than that of the reference strain *B. licheniformis* PWD-1 (ATCC 53757). Optimum keratinase production by this bacterium was achieved in 32 h using a minimum growth medium at 50 °C, pH 8.5 and containing 1.1% (w/v) feather meal. The molecular weight of the keratinase was $\approx 28 \text{ KDa}$ as determined by mass-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS). The MALDI-TOF MS spectrum of the tryptic peptides of this enzyme did not match any previously reported keratinases.

Conclusions: There are few reports on the evaluation of feather degrading ability of keratinases using highly resistant melanised feather. The keratinase reported here significantly degraded melanised feather in 48 h in the absence of reducing agents and may offer an environmentally friendly solution to the keratin and feather waste problem.

Background

The poultry processing industry produces a significant amount of feather waste; the United States and the United Kingdom generate about 4 billion pounds [1] and 150,000 tonnes (331 million pounds) [2] of feather waste per annum respectively. The disposal of such considerable quantities of waste by landfill requires large areas and the alternative option of incineration creates additional pollution burden. Neither method is economically viable or environmentally sustainable. In addition, feather wastes are potentially valuable raw materials for the production of nutrient-rich animal feed supplements and amino acids [3, 4]. Feather waste processing methods such as steam pressure, chemical treatment and feather milling are cost and labour intensive and may reduce the product's nutritional value by destroying important amino acids [3, 5].

Feather is composed of approximately 90% pure keratin; predominantly in the form of β -keratin [6]. The tertiary structure of β -keratin is highly organised: the cross-linking of disulfide and hydrogen bonds along with hydrophobic interactions stabilises the structure and together with its aggregated amyloid-like fibrillar form confer mechanical strength as well as chemical and enzymatic resistance [7- 9]. Interestingly, despite these highly stabilising characteristics, an increasing number of enzymes, isolated from certain environmental microbial strains are found to degrade feather keratin [10-12]. Enzymatic degradation of feather is particularly important because it provides an operational advantage over bacterial fermentation that requires controlled conditions for cell growth [13].

Keratinases have been found to efficiently hydrolyse keratin and degrade feathers into useful livestock feeds [4, 14, 15] and organic fertilizers [16]. In addition, keratinases are useful for removing hair from hides in the leather processing industry [17-19] and for the degradation of prions [20, 21]. Thus they have important applications in biotechnology and waste management.

A number of keratinase producing microorganisms (e.g. fungi [22], actinomycetes [23], *Streptomyces* [24], *Bacillus* [25] and *Pseudomonas* [26] species) have been isolated from various environmental sources such as soil, poultry farm wastes and raw feather [27, 28]. The great potential for keratinase in the biotechnology industry demands the most efficient and environmentally friendly sources be identified and exploited for maximum production. Farmyard wastes are characterised by a rich and diversified microbial ecology [24, 28, 29]. Thus they constitute an excellent source of prolific keratinase producing microorganisms. In the present work, a number of keratinase producing organisms from farmyard wastes

have been isolated and a prolific keratinase producer was identified and designated as *Bacillus licheniformis* N22. This strain produced a distinct MALDI-TOF MS spectrum which was different from that of the reference strain *B. licheniformis* PWD-1. The keratinase has a unique peptide spectrum and was able to significantly degrade melanised feather.

Results

Isolation and identification of feather degrading bacteria

Microbial strains from primary effluent, poultry and animal wastes were isolated on feather meal agar (FMA) plates. The proteolytic and keratinase activities of the isolates were determined by the casein agar assay and keratinase assay respectively.

The hydrolysis ring assay enabled the identification of microorganisms that have the ability to hydrolyse casein. The diameter of hydrolysed casein ring for the neat, 1:10; 1:100; 1:1000 dilutions of crude enzyme were 20 mm, 16.3 mm, 12.5 mm and 6.3 mm respectively (Figure 1). The casein ring diameter was inversely correlated to the dilution of crude keratinase (Pearson correlation = 0.761, $p=0.239$). Based on the casein ring analysis, thirty-two best performing microbial strains were isolated for further study.

Results from the keratin azure assay are presented in Figure 2. Of the thirty-two isolates strain number 22 produced the highest activity ($11.00 \pm 0.71 \text{ U mL}^{-1}$). Keratinase produced by the reference strain *B. licheniformis* PWD-1 (ATCC 53757) was also analysed; the activity of Strain N22 was 58% higher than PWD-1.

Isolate N22 was found to be a Gram positive rod bacterium displaying a number of characteristics similar to the *Bacillus* species listed in the Bergey's Manual of Determinative Bacteriology [30] (Table 1). Gene sequence analysis of the 16S rDNA revealed 99.93% homology with *B. licheniformis* ATCC14580. A phylogenetic tree which was generated by MicroSeqTM (Figure 3) showed that strain N22 was within the same branch of *B. licheniformis*. The characteristic peaks in the mass spectral fingerprints were stable, reproducible and different from those of the reference strain PWD-1 (Figure 4). We have isolated a new strain of *B. licheniformis* and designated it as *B. licheniformis* N22. It has been deposited with the National Collection of Industrial Food and Marine Bacteria (NCIMB): its ascension number is NCIMB 41708.

Optimisation of keratinase production and characterisation of keratinase

In order to optimise the enzymatic production of N22, the optimum conditional for microbial growth and enzyme production were investigated.

Keratinase production by *B. licheniformis* N22 occurred over a wide pH range (6-12), with the optimum lying between 8 and 9 over 32 h. Optimum substrate concentration and temperature for keratinase production were 1.1% (w/v) and 50 °C respectively. Keratinase production was greatly inhibited at acidic pH ($\text{pH} \leq 4$), substrate concentration above 1.1% and a temperature of 65 °C and above (Figure 5).

With respect to keratinase activity, the maximum activity of $11 \pm 0.71 \text{ U mL}^{-1}$ was attained at pH 8.5, feather meal concentration of 1.1% (w/v) and a temperature of 50 °C. Conditions that were averse to the production of this keratinase had similar effects on its activity.

The purified keratinase was analysed by MALDI-TOF MS and a single peak was obtained in the mass spectrum (Figure 6) showing that the molecular weight of this keratinase was approximately 28 kDa. The single peak depicts homogeneity of the sample. The activity of this keratinase was maintained at 11 U mL^{-1} after 8 weeks storage at 4 °C.

The peptide mass fingerprint of the purified keratinase is presented in Figure 7. The original peak of $\approx 28 \text{ KDa}$ was cleaved to generate a number of tryptic peptides. An overlap of trypsin-digested (blue) and non-trypsin digested (red) keratinase spectrum were used to establish the peptide peaks and matched against trypsin spectrum to identify any background peaks. A tryptic peptide search on Mascot Search Engine (Matrix Science) did not yield any match to previously reported keratinases.

Degradation of melanised feather

The catalytic activity of crude keratinase was investigated by its effect on melanised feather. Melanised feather was significantly degraded by crude keratinase (Figure 8). A mean reduction of about 80% in the weight of melanised feather ($52 \pm 2 \text{ mg}$ to $10 \pm 3 \text{ mg}$) was obtained when treated with crude extract for 48 h as compared to 12% ($52 \pm 2 \text{ mg}$ to $46 \pm 3 \text{ mg}$) reduction for control which was made up of crude keratinase that had been boiled at 100 °C for 30 minutes ($P = 0.038$; 2- Sample T-test).

Discussion

Since the first report on the isolation of extracellular alkaline serine protease from *Bacillus* sp. strain 221 [31], the search for industrially relevant proteases from different

environmental sources such as farmyard wastes [28] and sewage sludge [32] has been continuous. For mass screening of keratinase producing microorganisms, the casein-agar assay was a useful preliminary tool to detect proteolytic microorganisms. Casein is a highly stable and hydrophobic non-fibrous protein. It does not coagulate or easily denatured by heat but can be hydrolysed by certain proteases [33, 34]. These characteristics informed our choice of the method used in this study to identify proteolytic microorganisms that can hydrolyse casein and other structurally similar substrates such as keratins. The casein hydrolysis ring assay is similar to the method described by Tork et al [28] where the diameter of the clear zone was used to determine the microbial growth rate. In this study a correlation between the sizes of the hydrolysis rings and the protease catalytic activity was demonstrated (Figure 1). This confirms the hydrolysis ring assay as an efficient, cost and labour effective semi-quantitative method for measuring proteolytic activities. However, since not all proteases can degrade keratin, an evaluation of keratinolytic activity of potential keratinase producers should be confirmed by the keratin azure assay which is highly specific for determination of keratinase activity.

Of the thirty-two isolates, strain N22 demonstrated the highest keratinase activity and was selected for further study. It was identified as a Gram-positive, endospore-forming, mostly alkalophilic, mildly thermophilic and halotolerant bacterium (Table 1). The aerobic and mild and easily manageable growth conditions (37-60 °C and pH 6-10) make this bacterium an attractive candidate for biotechnological applications. Gene sequence analysis using 16S rDNA indicated that strain N22 is closely related to *B. licheniformis*. Other *B. licheniformis* are also known to produce keratinase [25, 35], but strain N22 differs from the well characterised *B. licheniformis* PWD-1 [25] as the former is unable to ferment lactose. Furthermore, these two strains produced distinct spectra when analysed by MALDI-TOF MS (Figure 4), suggesting N22 and PWD-1 are two different species. The crude keratinase produced by *B. licheniformis* N22 also expressed 58% higher activity than the keratinase from *B. licheniformis* PWD-1 on keratin azure substrate (Figure 2).

The interplay of various factors such as temperature, pH, substrate concentration, the nature and composition of carbon and nitrogen sources as well as the condition of inoculants, influences cell growth and survival, and the levels of protease synthesis in a microbial culture [36]. Thus, optimisation of these factors is important for an effective synthesis of biological entities such as keratinases. The optimum production of keratinase by strain N22 was achieved at a substrate (feather meal) concentration of 1.1%, pH 8.5 and 50 °C over 32 h. The most important limiting factors affecting keratinase synthesis appeared to be low pH ($\text{pH} \leq 4$) and high temperature (≥ 65 °C). As *B. licheniformis* is

mildly thermo- tolerant with its optimum temperature of 50 °C, it may be adapted for microbial composting of organic wastes.

Feather meal substrate induced keratinase synthesis by *B. licheniformis* N22 resulted in a 68% increase compared to its culture grown in Nutrient Broth (results not shown). This study confirmed that high substrate concentration reduced keratinase production (Figure 4) which was previously reported by Brandelli and Riffel [27], Wang and Shih [37] and Lin and Yin [38].

Approximately 40% of total cost of enzyme production is due to the cost of growth substrates [39]; the use of low cost and readily available feather meal or chicken feather substrate will enable the sustainable production of strain N22 keratinase on an industrial scale. In addition, the use of feather waste as growth substrates for keratinase producing microorganisms will invariably serve as an efficient way of managing the significant amounts of chicken feather wastes produced by the poultry processing industry [40].

The molecular weight of *B. licheniformis* N22 keratinase was found to be \approx 28 KDa MALDI TOF-MS (Figure 6). The single mass peak obtained by MALDI TOF-MS indicates that the purified keratinase is homogeneous and monomeric (has no subunits). This molecular weight is very close to that of *Bacillus pseudofirmus* FA30-10 (27.5 KDa; [41]); it is different from that of *B. licheniformis* PWD-1 (33 KDa; [42]). This suggests that *B. licheniformis* N22 keratinase is a distinct entity. Peptide mass fingerprint of trypsinised N22 keratinase (Figure 7) did not produce any matches in the Mascot Search Engine (Matrix Science) confirming that this is a novel keratinase: it has not been previously characterised and documented.

The purified keratinase was stable at 4 °C after 56 days compared to the keratinase produced by *B. licheniformis* PWD-1 which had been reported to lose a quarter of its activity after 19 days when stored at the same temperature [43]. Keratinase from *Streptomyces fradiae* also retained its activity for several weeks at 4 °C when stored at pH 7 but rapidly loses its activity at pH 8.5 [44]. Feather degradation generally occurs under alkaline conditions as a result of deamination [45]; this is within the optimum pH range for the keratinase reported in this study. Thus it appears to have a more robust potential for biotechnology exploitation especially in feather waste management.

In the present study, melanised feathers were cleaned by agitation with sterile distilled water to ensure that they remained structurally uncompromised and free from microbial contamination [46]. Sterilisation by autoclaving denatures feather keratin and causes the

feather to be susceptible to proteolytic attack as they lose their insolubility and resistance to enzymatic degradation [47, 48]. There are relatively few reports on the evaluation of keratinolytic activity using melanised feather substrate. Rather, in most reported feather degradation studies, the feather substrates are usually young, downy white feathers [27, 49, 50]. Notably, melanised feathers are generally more resistant to enzymatic attack than white feathers due to the presence of the pigment melanin which binds to and inhibits keratinases [48, 51] as well as other enzymes [52]. The cell-free crude keratinase of *B. licheniformis* N22 significantly degraded melanised feather ($p = 0.038$; Figure 8). The residue (mainly rachises) was soft and brittle and had to be handled with great care during drying and weighing. The rachis constitutes most of the total feather mass and has been reported to resist degradation by *B. licheniformis* [53]. Our study has shown that at optimum conditions the reported enzyme demonstrated a much more profound catalytic activity than any of the known keratinases.

Feather degradation by keratinases has been mostly performed in the presence of suitable reducing agents such as live cells or chemical reductants [27, 13, 54]. Cell-free keratinases of *Streptomyces* BA₇ [55], *Streptomyces* S7 [56] and *B. licheniformis* ER-15 [19] have been reported to degrade feather in 24 h, 97 h and 12 h respectively. However, only 10-20% degradation is reportedly achieved by cell-free keratinases in the absence of reductants [57]. Most purified keratinases are unable to effectively degrade native keratin for reasons which include: (a) the high degree of disulfide bonds in the keratin molecules [58, 59] and (b) the removal, during purification, of fermentation culture constituents that are capable of reducing or breaking disulphide bonds [49]. The latter suggests that the presence of a consortium of enzymes may be required to enhance feather keratin degradation [26]. In contrast, the purified keratinase of *B. licheniformis* N22 was able to significantly degrade melanised feather in the absence of reducing agents.

Conclusions

We have isolated a novel strain of *B. licheniformis* from farmyard waste which has a profound ability to produce keratinase of prolific activity: it significantly degraded melanised feather within 48 h in the absence of reducing agents. The keratinase has a trypsin peptide MALDI-TOF MS spectrum not previously reported. By optimising its production and activity, this keratinase has great a potential in providing new, rapid and cost-effective methods for the management of feather and other wastes of similar molecular structure.

Methods

Isolation and identification of microorganisms

Minimum Growth Medium (MGM) containing (in gL⁻¹): NaCl, 0.5; KH₂PO₄, 0.7; K₂HPO₄, 1.4; MgSO₄·7H₂O, 0.1; pH 7 [37] was used as the basic growth medium.

Microbial strains were isolated from primary effluent (Deephams Sewage Treatment Facility, Thames Water, Edmonton North London, UK) and poultry/animal wastes (A.K Woods Poultry Farm, Fold Farm Partners and Leamon Pig Farm Ltd, UK). Sample of wastes (1 g) were serially diluted in a quarter-strength Ringer's solution (Oxoid, UK), plated out on feather meal agar [10% commercial feather meal (Chettles Ltd, UK) in MGM and 1% agar] plates and incubated at 30 °C, 37 °C or 50 °C for 24, 48 and 72 h. Single colonies were passaged twice on feather meal agar (FMA) plates to obtain microbial monoculture. The purified isolates were grown in Nutrient Broth (Oxoid, UK) and stored in liquid nitrogen in 0.5 mL aliquots.

Screening for proteolytic and keratinolytic activity

A number of wells were aseptically punched onto a casein agar (Oxoid, UK) plate surface, using a sterile 7 mm diameter puncher. The wells were inoculated with 100 µL of the bacterial culture and incubated at 30 °C, 37 °C or 50 °C for 24 h. The diameters of the hydrolysis rings produced by the isolates were measured; those that produced the highest diameters were selected for further examination. A validation experiment for hydrolysis ring assays was carried out using the crude enzyme extract at 1:0; 1:10; 1:100; and 1:1000 dilutions.

Strains selected for high activity were further cultured in 50 mL MGM containing 1.1% feather meal. The crude enzyme extract was recovered by centrifugation at x 4750 g for 20 min in a Rotina 420R Centrifuge (Hettich, Germany). The supernatant was collected and vacuum filtered through 0.45 µm sterile membranes (Pall Corporation, USA).

Keratinase activities of the crude enzyme extracts were determined using the method described by Letourneau *et al* 1998 [60]. One unit of keratinase activity (U) was defined as the amount of enzyme producing an absorbance change of 0.01 units (A₅₉₅). The keratin azure substrate was treated prior to the assay to ensure uniformed release of the azure dye and reliable absorbance measurement. Briefly, keratin azure was incubated with 5 times the displaceable volume of distilled water for 1 h at 50 °C and 250 rpm in a rotary incubator. The resulting keratin azure was washed thrice with sterile distilled water by shaking vigorously for 1 min and allowed to dry at 30 °C in an oven.

Initial identification of the microorganism was performed based on their morphological, physiological and biochemical characteristics [30]. Molecular identification using MicroSeqTM based on the 16S rDNA full gene sequence was performed at the National Collections of Industrial Food and Marine Bacteria (NCIMB), UK.

To generate the microbial mass spectral profile, a single bacterial colony was selected and the whole cell protein was extracted with 1 mL mixture of acetonitrile and 70 % formic acid (1:1 v/v). The extracted protein sample (1µL) was conjugated with 1µL of the α -cyano-4-hydroxycinnamic acid matrix (Sigma, USA) and analysed by the MALDI-TOF MS (AXIMA CFR, Shimadzu Biotech).

Keratinase production and characterisation

Keratinase production was optimised using the following parameters: temperatures at 37 °C or 50 °C; pH 7, 8.5 or 10) and feather meal concentration of 0.8%, 1.1% or 1.4% (w/v). All experiments were carried out in triplicates.

Cell-free crude keratinase extract was concentrated using a Centriconplus-70 centrifugal filter device (Millipore Inc.) with a 10 KDa molecular weight cut-off and purified using a HiTrap Blue HP purification column (GE Healthcare Bio-sciences). To determine the stability of the purified keratinase, samples were stored at 4 °C and its activity reassessed after 8 weeks.

The molecular weight of the purified keratinase was determined by MALDI-TOF MS (AXIMA CFR, Shimadzu Biotech).. The purified keratinase sample (1 µL) was conjugated to sinapinic acid matrix (1 µL) (Sigma-Aldrich, USA) on a sample metal plate and allowed to crystallise at room temperature.

The purified keratinase was digested with 0.1mg/ml of trypsin (Sigma, UK) at 37 °C for 1 h to obtain the peptide mass fingerprinting. The resulting MALDI-TOF MS spectrum was searched on Mascot Search Engine (Matrix Science) to identify any possible matches.

Degradation of melanised feathers

Melanised feathers were washed with sterile distilled water and tested for microbial contamination. These were dried (50 °C) to obtained dry weight, suspended in distilled water and incubated at 50 °C with crude or denatured crude keratinase for 48 hours in a rotary incubator (Stuart, UK) at 250 rpm. The differences in weights indicated the extent of degradation of the feather substrates. The data were analysed using a two-sample t-test for their significance at the 95% confidence interval.

Authors' contributions

EAO contributed to the research design, carried out experiments, participated in data analysis and interpretation and drafted the manuscript. HG contributed to the design of experiments and in revising the manuscript. OOA contributed to conception; design of research, data interpretation and in revising the manuscript. DP contributed to the conception and design of research, participated in data analysis and presentation and in revising the manuscript. All authors' read and approved the final manuscript.

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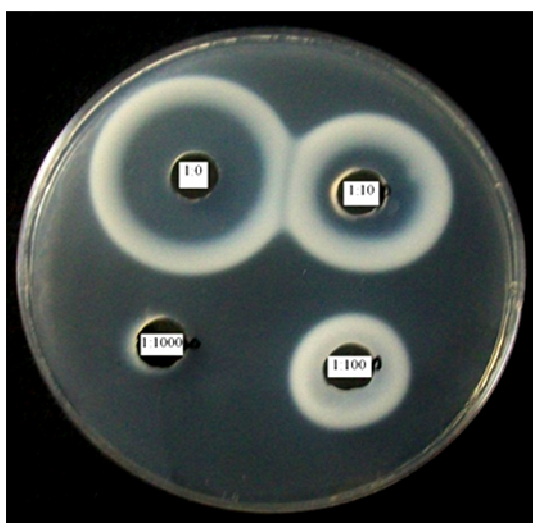
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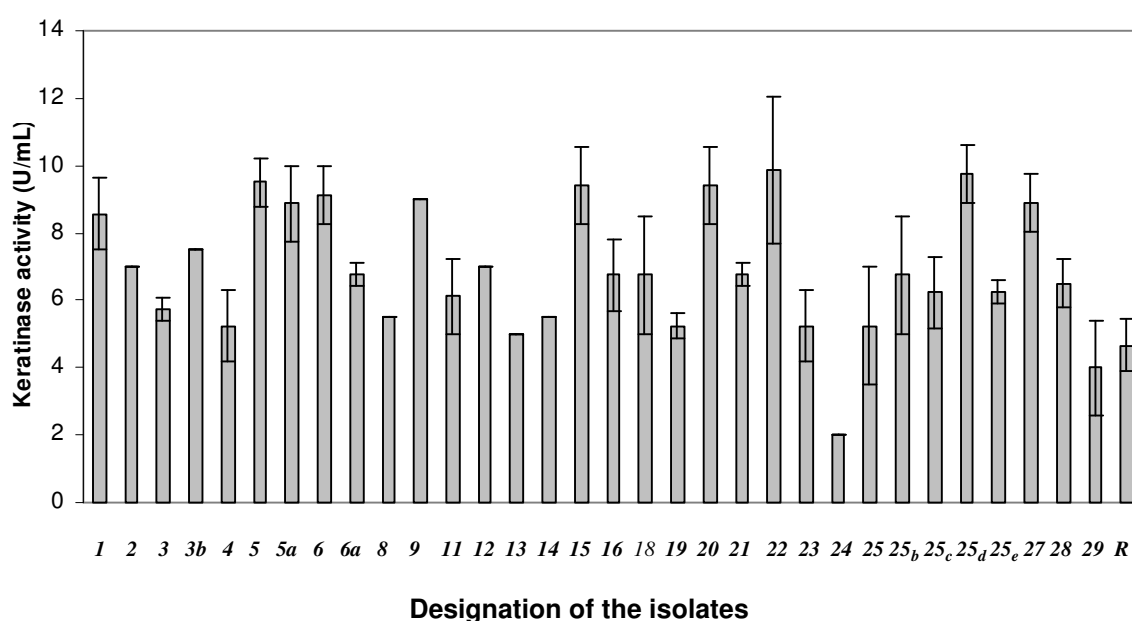
Figures

Figure 1 - Casein hydrolysis ring assay



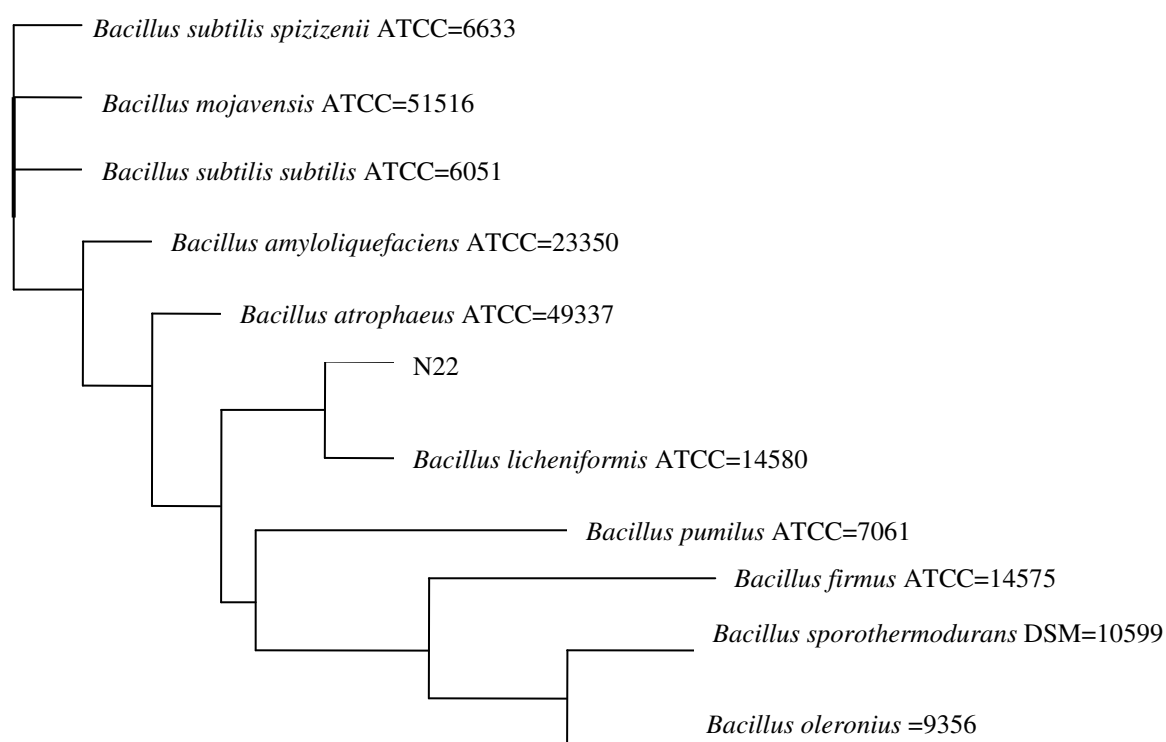
Casein agar plate showing the relationship between keratinase activity and the casein hydrolysis rings. Clockwise from top left are inoculations with keratinase dilution at 1:0, 1:10, 1:100 and 1:1000. The casein agar plate was incubated at 50 °C for 24 h.

Figure 2 - Keratinase activity for microbial isolates



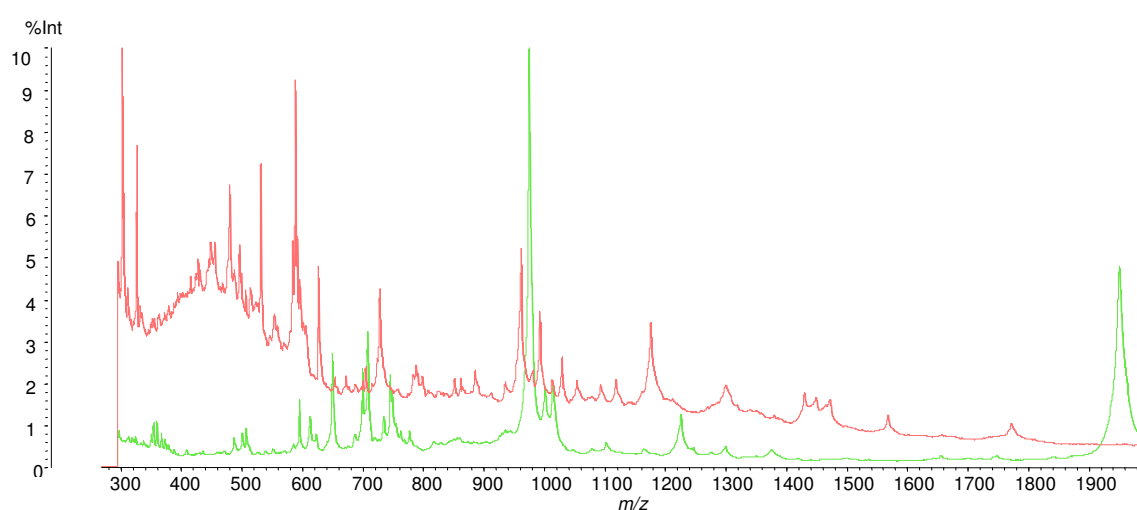
Keratinase activity ($\text{U mL}^{-1} \pm \text{SD}$) for the 32 microbial isolates as measured by the keratin azure assay. The bacterial cultures were incubated in MGM supplemented with 1.1% feather meal for 48h, at their isolation temperatures: strains 3-9, 13, 25 d. The reference strain *Bacillus licheniformis* PWD-1 (labelled R) were incubated at 50 °C while all the remaining strains were grown at 37 °C.

Figure 3 - Phylogenetic tree showing isolate N22 within a cluster of *Bacillus licheniformis* strains



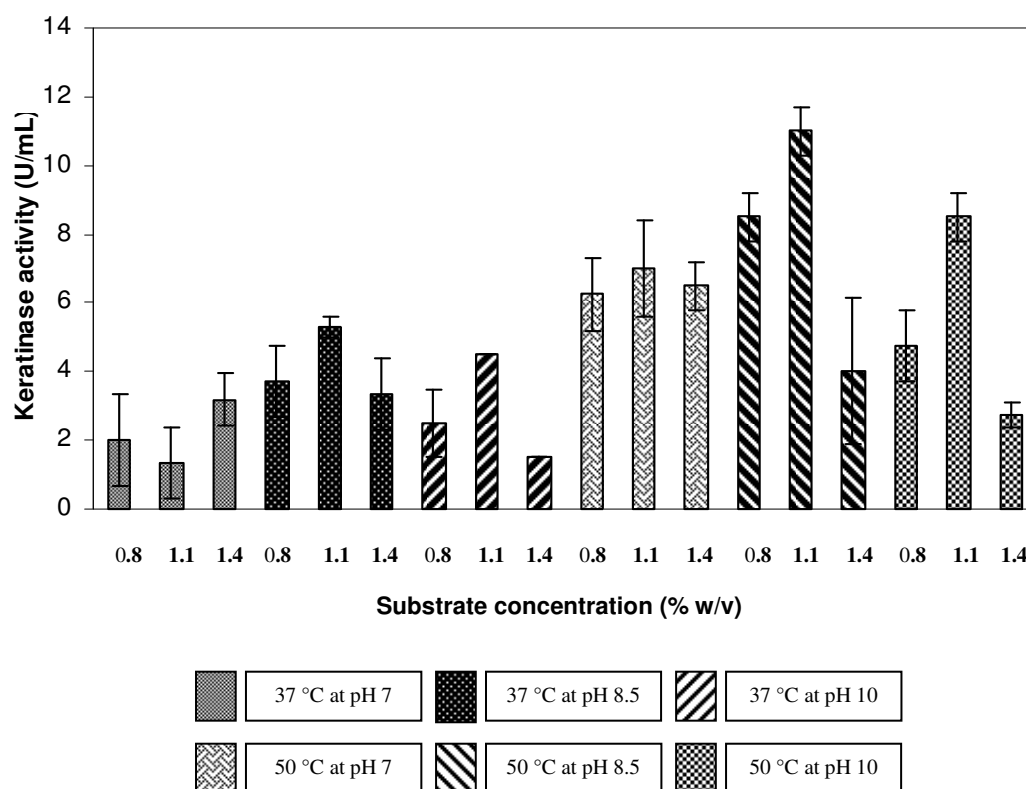
Phylogenetic tree (built with MicroSeqTM database, NCIMB, UK) showing the top 10 hits generated from the 16S rDNA full sequence analysis of *Bacillus licheniformis* N22 within a cluster of *Bacillus licheniformis* specie.

Figure 4 – The mass spectral fingerprints for *Bacillus licheniformis* N22 and *Bacillus licheniformis* PWD-1



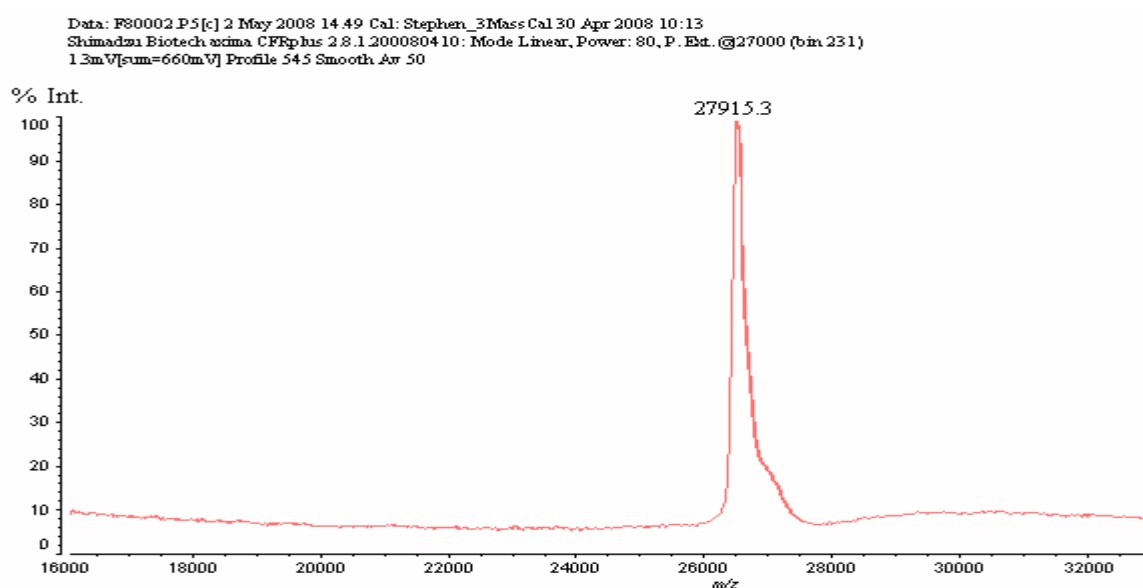
The MALDI-TOF MS spectra showing distinct protein peaks (fingerprints) for strains N22 (in red) and PWD-1 (in green).

Figure 5 - Optimisation of fermentation culture for keratinase production



The effects of temperature, pH and substrate concentration, on keratinase production by *B. licheniformis* N22. Optimum production was achieved at 50 °C, pH 8.5 and a substrate concentration of 1.1% (w/v). Each pattern represents a specific pH and temperature (°C) used. The X-axis denotes the concentration (% w/v) of the feather meal in the medium.

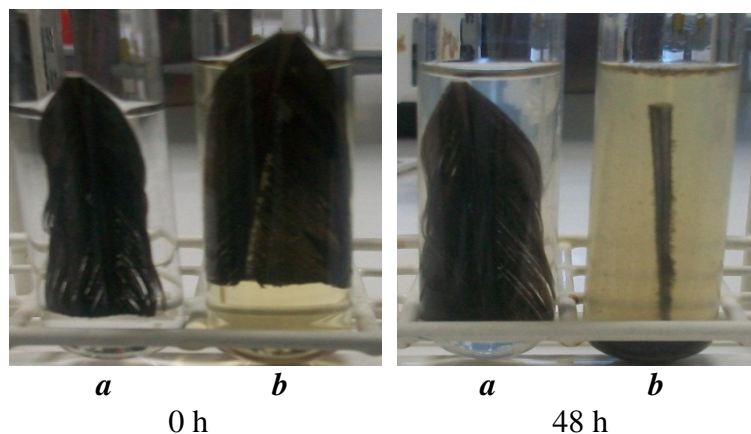
Figure 6 – Molecular weight of *B. licheniformis* N22 keratinase



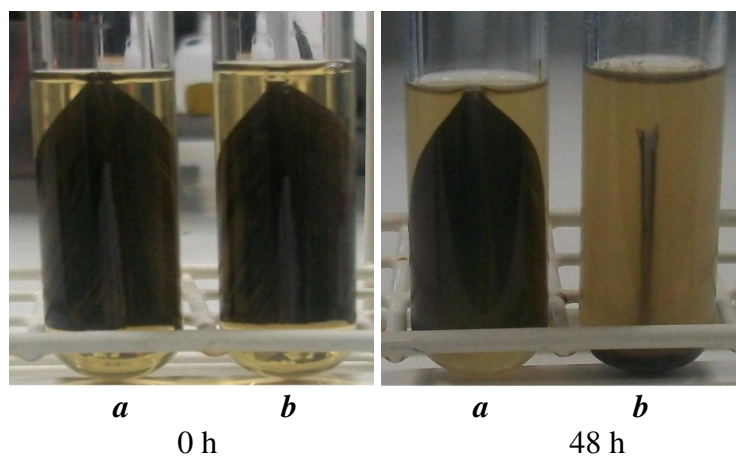
MALDI-TOF MS spectrum showing molecular weight of keratinase as ≈ 28 KDa. Single peak depicts homogeneity of the sample.

Figure 8 - Degradation of melanised feather by crude keratinase showing the state of feather at 0 and 48 h.

(A)



(B)



Degradation of melanised feather by crude keratinase showing the state of feather at 0 and 48 h. In photographs labelled (A), the feathers in test tubes 'a' and 'b' were incubated in 10 mL of distilled water and dilute crude keratinase (2 mL in 8 mL of distilled water) respectively. In photographs labelled (B), the feathers in test tubes 'a' and 'b' were incubated in 10 mL of denatured crude keratinase (boiled for 30 min) and undiluted crude keratinase respectively.

Table

Table 1 - Morphological, physiological and biochemical characterisation of isolate N22

Tests	Response
Colony morphology	Rod-shaped, rough edge, mucoid, domed surface, creamy-white colour
Gram stain	+
Endospores formation	+
Anaerobic growth	-
Motility	+
Oxidase reaction	+
Catalase reaction	+
<i>Glucose</i>	+
<i>Lactose</i>	-
<i>Iron</i>	-
Reduction of nitrates to nitrites	+
Voges-Proskauer (VP)	+
Indole production	-
Arginine DiHydrolase	+
Urease	+
Hydrolysis of casein	+
Onset of growth on nutrient agar plate (37 °C)	9 h
(50 °C)	5 h
Growth in nutrient agar at NaCl conc. 2-12 %	+
Growth in nutrient broth at pH 6-12	+